THE UNIVERSITY
of EDINBURGH

Effects of metal speciation on metal plant dynamics in the presence of plant growth promoting bacteria

Submitted by
Nyekachi C. Adele

A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

The University of Edinburgh

2016

Declaration

- The thesis has been composed by the candidate named above.
- All work included is my own original work, except where due reference is made.
- This work has not been submitted for any other degree or professional qualification except as specified above.

Signature……...

Date……

Lay Summary of thesis

Zinc is essential for plant growth as it acts as a component of a larger number of enzymes. However, high concentrations of zinc in soil are also toxic to plants, although the toxicity depends somewhat on the chemical form in which it exists in the soil environment. Traditionally, soil contamination by zinc has been linked to mining activities but modern advances in nanotechnology has led to concerns that nanoparticulate industrial forms such as ZnS and ZnO may constitute another important source of soil contamination, prompting calls for understanding how these might affect plant health. This thesis investigated whether the form (speciation) to which plants are exposed affects zinc uptake by plants as well as plant health and the efficiency of metal remediation by the plant. Pot experiments using Brassica juncea (L) Czern, which is known to accumulate large concentrations of zinc (hence known as a hyperaccumulator) were conducted to assess the effect on plant growth in soil with elevated Zn concentrations in the form of soluble Zn or as ZnS and ZnO nanoparticles. Additional experiments investigated the role of soil bacteria on both plant health and zinc accumulation in plant tissues. Measures of plant health included plant height, number of leaves, root biomass, shoot biomass as well as chlorophyll content. Spectroscopic techniques using high energy x-rays were combined with microscopic imaging to determine the form and distribution of in root tissues at the end of each growth experiment. Measurements of plant height, number of leaves, root length, plant biomass and leaf chlorophyll content of Brassica juncea grown in 600 mg Zn kg^{-1} of $ZnSO_4$, ZnS and ZnO nanoparticles showed that $ZnSO_4$ was the most toxic to plants compared to ZnS and ZnO nanoparticles treatments. Soil bacteria promoted plant growth, biomass and zinc accumulation in plant roots and shoots. Spectroscopic analysis showed that the observed reduction in toxicity in the presence of bacteria was due to a change in the form in which zinc occurred in plant tissues, with zinc being bonded to sulfur-bearing proteins synthesised by the bacteria. This change occurred regardless of the initial form in which zinc was added to soil, suggesting a universal mechanism by which bacteria help to detoxify metals to plants. By also assessing Zn speciation changes across the soil-rhizosphere-plant interface, this study established that bacteria modified Zn speciation at the rhizosphere. The entire study emphasises on the role of metal speciation as a major determining factor in understanding plant tolerance and has implications for potential application of Brassica plants in decontaminating soils through a process called phytoremediation.

Abstract

Excessive metal deposition in soil is of major concern to the environment due to the toxicity of metals to animals and plants. Since metals do not degrade, reducing risk of exposure relies in either removing the metals from soil, or changing their speciation which leads to changes in bioavailability, mobility and toxicity. Plants have been shown to provide a cheap alternative to chemical methods for both removing and changing metal speciation, particularly when augmented with plant growth promoting bacteria. The focus of this thesis was to investigate whether the form (speciation) in which a metal contaminant is introduced to soil affects both plant health and the efficiency of metal remediation by the plant, using the well-known hyperaccumulator *Brassica juncea (L.)* Czern and zinc (Zn) as the metal contaminant. This study also examined the role of plant growth promoting bacteria in changing metal speciation, impact on metal toxicity and phytoremediation efficiency. *Brassica juncea* was grown in pots containing soil spiked with equal amounts (600 mg Zn kg^{-1}) of soluble Zn ($ZnSO_4$) and nanoparticulate ZnS and ZnO. Plant height, number of leaves, root length, plant biomass and chlorophyll content of *Brassica juncea* were used to assess Zn toxicity. Zn localisation and speciation in soil and plant tissues was studied using transmission electron microscopy (TEM), synchrotron micro-X-ray fluorescence elemental mapping (μXRF) and synchrotron X-ray absorption spectroscopy (XAS). Growth parameters showed that $ZnSO_4$ was the most toxic form of Zn whilst ZnS and ZnO effects were not statistically different. These differences were linked to differences in Zn content in root and shoot biomass, which was higher in $ZnSO_4$ treatments. Inoculation with *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* enhanced plant growth, Zn concentration in plant biomass and translocation of Zn in all Zn treatments. XAS analysis showed that Zn speciation was altered in roots of plants inoculated with bacteria, with Zn cysteine as the most dominant form of Zn in all inoculated Zn treatments, suggesting a role for cysteine in ameliorating Zn toxicity. By also assessing Zn speciation changes across the soil-rhizosphere-plant interface, this study established that *Rhizobium leguminosarum* modified Zn speciation at the rhizosphere. Through this thesis work, metal speciation

is a major factor in determining the efficiency of metal phytoremediation and plant tolerance. Hence, this research provides useful information on Zn speciation which will contribute to effective implementation of Zn phytoremediation.

Dedication

With great joy and affection I dedicate this dissertation

To my loving father, Chief G.O Adele (JP) who would have loved a copy of this dissertation but did not get to see my completed dissertation. Continue to rest!!

To my children Daniel and Daisy, who stormed my life with love, happiness and changed me into the woman I have become.

Acknowledgement

I wish to express my sincere appreciation to remarkable individuals who contributed to this thesis and supported my work during this amazing journey.

Foremost, I would like to thank my supervisor, Dr Bryne Ngwenya, for his inspirational discussions, patience, enthusiastic guidance, training and mentorship. He has been a great advisor outside of the academic world and has keen interest in my overall wellbeing and that of my family throughout my PhD. My sincere thanks go to my co-supervisor Professor Kate Heal, she provided me an excellent guidance, ever ready with brilliant ideas, critical input and honest advice and I am extremely grateful for having had the opportunity to do my PhD under her supervision. To Professor Fred Mosselman, from whom I have learnt to use synchrotron X-ray absorption spectroscopy, his expert advice and assistance into the world of synchrotron XAS is exceptionally appreciated.

My appreciation goes to the Rivers-State Sustainable development Agency (RSSDA) Rivers-state, Nigeria for awarding me a full scholarship to fund my entire PhD programme. Without this funding opportunity, I would have struggled more financially. Many thanks go to staff and management of Diamond Light Source, Didcot (UK) for successfully awarding two beamline times and access to synchrotron beamline i18 and B18 through projects SP9151 and SP10429. Chapter 6 and 7 of this thesis probably would not exist.

I would like to acknowledge the kind assistance of the Dr Sophie Haupt and team for their kindly guidance and for providing me with the necessary equipment for growing plants at the glasshouse. I must thank the faculty and staff of Geoscience department, who added much delight throughout my program, I will cherish my experiences.

I feel privileged to have made friends, which are too many to name. I am indebted to my colleagues and friends for providing a fun filled environment. Special thanks to Temi, for helping me with XAS analysis and for many rounds of useful discussions on

my project. Tanaz, Lige, Claire, Hannah for all the friendly chats, encouragement and wonderful memories.

My deepest love and admiration go to my parents for raising me, instilled admirable qualities and good foundation with which to meet life. Their unconditional love, prayers, encouragement at every stage of my personal and academic life has kept me going. I deeply miss my father, Chief G.O Adele (JP), who is not with me to share this joy.

A special thank goes to my siblings (Nora, Miriam, Sylvia, Juliet, Marshall, Charles and Queeneth) who have given me their unequivocal support, encouragement, prayers and made me feel emotionally close when we were physically apart. You have all given me so much, in so many ways and am very grateful to have you with me on this journey. Also, I thank my in-laws for their generous support and belief in me.

To my husband, Chile, for been a great supporter, patient, instrumental in instilling confidence and for the wonderful life that we share. To my precious kids, Daniel and Daisy, in your smiles and cuddles I renew strength. Thanks for staying healthy and being wonderful!

Above all, I owe it all to the Almighty God for granting me life, strength and wisdom to undertake this research and enabling me to its completion.

Abbreviations

ANOVA	Analysis of variance
Cys	Cysteine
DEFRA	Department for Environment, Food and Rural Affairs
EPA	Environmental protection agency
ICP-OES	Inductively coupled plasma optical emission spectrometry
L	Litre
NPs	Nanoparticles
P	Probability
PGPB	Plant growth promoting bacteria
Phy	Phytate
SEPA	Scottish Environmental Protection Agency
SO_4^{-2}	Sulfate
TEM	Transmission electron microscopy
UK	United Kingdom
USEPA	US Environmental Protection Agency
XANES	X-ray absorption near-edge structure
XAS	X-ray absorption spectroscopy
XRD	X-ray powder diffraction
Zn	Zinc
ZnO	Zinc oxide
ZnS	Zinc sulphide

Table of contents

List of Figures

List of Tables

Chapter 1

1 Project rationale

Increased metal concentrations in soils negatively influence plant health (Long et al., 2002; Duquene et al., 2009). Zinc, as an essential element influences several metabolic processes of plants and occurs naturally in soil (Davies and Jones, 1988). Soils may become contaminated by the accumulation of zinc through usage of Zn nanomaterials (Tourinho et al., 2012), emissions from rapidly expanding industrial activities, mining and smelting, land application of fertilizers, sewage sludge, pesticides, and atmospheric deposition (Singh et al., 2014). Zinc, as a typical metal is non-degradable and its total concentration in soils persists for a long time after introduction (Koopmans et al., 2008; Pajuelo et al., 2011; Singh et al., 2014). Thus, there is a need to implement appropriate remedial measures for Zn contaminated environments. Conventional remediation methods such as electrochemical removal, pump and treat, amongst others (Khan et al., 2004; Sharma et al., 2016) are prohibitively expensive and damaging to the soil structure and environment (Batty and Anslow, 2008; Bokhari et al., 2016). An alternative that incorporates the use of plant and microbes have been recently proposed to have potential for extracting or removing toxic metals from soil (Johnson and Singhal, 2011). Phytoextraction is a type of phytoremediation that is increasingly being used as an environmentally sustainable remediation technique which involves the use of plants to extract toxic metals from soil (Reichman, 2007). However, most plants suffer from toxicity of the metals being treated, and apart from specific species adapted to hyperaccumulate certain metals, their efficiency for metal removal is critically dependent on growth rate (Khan, 2005). *Brassica juncea (L) Czen* is a hyperaccumulator in which Zn accumulation occurs in shoots (Adediran et al., 2015). In the rhizosphere surrounding the plant roots, complex interactions occur between the root and the soil which influence Zn availability and hyperaccumulation (McGrath et al., 1997; Zhoa et al., 2012). Phytoextraction of Zn contaminated soil has been widely studied (e.g. Vamerali et al., 2010; Gomes et al., 2013). Studies have shown enhanced Zn

uptake, translocation and hyperaccumulation in *Brassica* species through the addition of either chelating agents (Ebbs and Kochian, 1998; Turan and Esringui, 2007) or plant growth promoting bacteria (PGPB) (e.g. Adediran et al., 2015; 2016; Ma et al., 2014). Several studies have shown the positive influence of metal-resistant PGPB on metal phytoextraction and in protecting plants from metal toxicity (Smical et al., 2008; Adediran et al., 2015). The mechanism by which this influence occurs might include secretion of ligands such as siderophores, indole acetic acid, and ACC deaminase or by mutualistic associations with bacteria (Ahmed and Kibret, 2014). Although the mechanisms involved are not clear, bacteria can also affect metal speciation through changes in valence state of the metal and by decreasing or increasing mineral solubility. The extent of assimilation of metals from soil depends on whether the metals are in a form that can be absorbed by plants (Maladrino et al., 2011).

To date, most studies regarding Zn (and other metallic contaminants) phytoremediation have focussed on soluble forms of the metal. However, some of these metals are increasingly finding their way into the environment in the form of engineered nanoparticles. Nanoparticles (NPs) are materials with at least a dimension between 1 and 100 nm (ASTM, 2012). As a novel and emerging class of products, there has been great concern about the potential for NPs uptake and translocation by plants (Zhang et al., 2015). Studies reviewed by Gardea-Toresdey et al. (2014) indicated that metal NPs (i) translocate to plant shoots and accumulate in the fruits/grains of agricultural crops, (ii) increase concentrations of the component metal in plant tissues, (iii) induce physiological, biochemical, and genetic modifications that may have detrimental or beneficial effects on the agronomic traits, yield, and productivity of plants, and (iv) induce modifications in the nutritional value of food crops. However, these studies have not conducted systematic comparisons of nanomaterials with soluble metals. There also appears to be no data regarding ZnS nanoparticles, one of the more prevalent forms in mining-impacted environments.

Knowledge of the speciation of metals, including in NPs is critical to achieve better understanding of the environmental impact of metals as speciation influences the bioavailability, mobility and toxicity of metals in plants (Shahid et al., 2012).

1.1 Research gaps and project aims

From the literature survey (see Chapter 2), the following research gaps have been identified that require further investigation.

(i) With the exception of those that look at the field environment, most pot experiments use soluble salts of heavy metals as a source of contamination. However, in most naturally and anthropogenically contaminated soils (e.g. mining-related pollution), contamination is likely to be in the form of minerals which must be dissolved before the metal can be taken up by plants. A fundamental question therefore is what mechanisms might lead to metal bioavailability to plants from solid matrices. These might include secretion of exudates such as amino acids, organic acid or mutualistic associations with bacteria.

(ii) Increasing use of NPs introduces an additional dimension to the form in which metal contamination occurs in soils. Because of their size, the potential exists that plants will assimilate nanoparticle- based metals both in solid form and in soluble form but only a limited number of studies have investigated this problem. In particular, nanoparticles potentially represent a transition between soluble and mineral-based metal bioavailability.

The primary aim of this research was to test whether the metal contaminant form (speciation) to which plants are exposed affects both (a) plant health and (b) the efficiency of metal remediation by the plant. A secondary aim was to test whether inoculation with bacteria (a) changes metal speciation and (b) whether these changes impact metal toxicity and phytoremediation efficiency in plants.

1.2 Research Hypotheses

The general hypotheses tested in this research are below:

1. Plant growth and phytoextraction abilities will differ in respect to the speciation of zinc they are exposed to in soil.

2. Zn uptake, accumulation and distribution in *Brassica juncea* will be dependent on the forms in which Zn was exposed in soil.

3. *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* will promote the growth of *Brassica juncea* under different Zn contamination compared to uninoculated controls based on their ability to confer multiple plant growth promoting abilities and ameliorate Zn toxicity in plants.

4. There will be differences in Zn speciation in inoculated and uninoculated *Brassica juncea* roots exposed to different Zn species in soil in order to promote phytoextraction.

5. There will be Zn speciation changes across the soil-rhizosphere-plant interface and *Rhizobium leguminosarum* (bv) *trifolii* will modify speciation across the soil-rhizosphere-plant interface of Brassica juncea (L.) Czern.

1.3 Outline of the thesis

This thesis consists of eight chapters.

Chapter 1 provides a general introduction to the research topic and knowledge gaps, identifies the research aims and objectives, and outlines the thesis structure. Chapter 2 comprises a literature review of the research conducted on metal speciation, nanoparticles, and phytoextraction and plant growth promoting bacteria. An overview of the research methods and design and their justification, including materials, equipment and experimental techniques, is provided in Chapter 3.

Chapters 4, 5, 6 and 7 represent the results and discussion of the experiments conducted to answer the research questions. Each of these chapters is presented in the format of a draft manuscript paper, comprising a brief introduction to the specific study, specific aims, materials and methods, presentation of results, discussion and conclusions.

Chapter 4 reports on a preliminary short study that investigated the Zn resistance patterns of selected PGPB to soluble Zn and Zn nanoparticle exposure. In this chapter, phytotoxicity differences due to soluble Zn and metal nanoparticles on germination and early seedling growth of *Brassica juncea* are also presented. The results of this chapter informed the choice of experimental materials and design in the plant growth experiments reported in Chapters 5 to 7. Chapter 5 examines the effect of different Zn forms on plant growth characteristics, uptake, translocation and accumulation in shoots of *Brassica juncea* (L.) *Czern* grown in Zn contaminated soil without the addition of PGPB. Chapter 6 examines the role of plant growth promoting bacteria on plant growth and its effect on the mechanisms of uptake, translocation and hyperaccumulation of different Zn forms in *Brassica juncea* (L.) *Czern.* Chapter 7 focuses on the differences and changes in Zn speciation between bulk soil, the root-plant interface in the rhizosphere and plant tissue following growth of *Brassica juncea* (L.) *Czern* in Zn contaminated soil with and without plant growth promoting bacteria. Chapter 8 completes the thesis, presenting an overview

of the key research findings in the form of a conceptual framework, recommendations for future work.

1.4 Significance of the study

The research reported in this thesis has potential wider significance in a number of ways:

- Remediation of metal contaminated sites is a complex problem. However, gaining a better understanding of the role of metal speciation in contamination scenario will minimise remediation costs, provide vital information on the potential hazard (mobility and toxicity) and allow identification of acceptable solutions to metal contamination.
- Results from this research project will provide a data-base to the industry, academia, and general public on phytoremediation technology and allow identification of techniques to investigate in more applied settings.
- It will contribute to the on-going evidence based on the potential risk that nanomaterials might pose to the environment and human health.

Chapter 2

2 Literature review

2.1 Introduction

Soil is the unconsolidated layer of the Earth's crust, composed of mineral particles derived from underlying geological materials, organic matter, organisms, air and water (Kabata-pendias et al., 2004; Certini and Ugolini, 2013). Soil provides essential ecosystem services, including nutrient cycling, carbon storage, water regulation, and food and fibre production (Kabata-pendias et al., 2004; White, 2013). Over several years of industrialization, soil contamination has become increasingly common in world with persisted toxic compounds, heavy metals, nanoparticles, radioactive materials, chemicals, salts build –up in soils (Khan et al., 2008; Guo et al., 2014). Contamination can seriously affect soil's ability to perform some of its key functions in the ecosystem. Thus the presence of substances that are available to cause significant harm to organisms needs to be considered (Semple et al., 2004).

This chapter reviews relevant academic research publications that would be helpful in understanding the subject under investigation and provides key definitions in understanding the entire thesis. This review is focused on heavy metal and nanoparticles soil contamination. Specifically, it addresses pertinent topics on contaminant environmental fate, speciation (bioavailability, mobility and toxicity) in the soil, including their effects in plants. The subsequent section specifically addresses the remediation of soil contaminated environments and the roles of plant growth promoting bacteria (PGPB) in soil contaminated environment.

2.2 Heavy metals

Metals account for about two thirds of all the elements and about 24% of the mass of the Earth. Most metals on Earth occur either in their pure state or in combination with other elements (e.g. as oxides, sulphides, carbonates and silicates). Heavy metals are a subset of the elements that are characterised by their metallic properties (such as; ductility, malleability, conductivity and high density). Heavy metals have been defined based on a number of factors, including chemical properties, toxicity, density, atomic weight or atomic number (Duffus, 2002).

Examples of heavy metals are aluminium, antimony, arsenic, barium, cadmium, cobalt, chromium, copper, iron, nickel, lead, manganese, molybdenum, selenium, zinc, titanium, vanadium amongst others. Some metals such as Hg, Cd, As, Se and Pb are not important or essential as they do not perform any physiological functions in plants while Zn, Ni, Mo, Mn, Fe, Cu and Co have known physiological importance as they are required for plant metabolism and growth (Gaur and Adholeya, 2004).

2.2.1 Metal contamination in soil and water

Soil (terrestrial ecosystem) and sediments (aquatic ecosystem) are the major sinks for heavy metal contaminants arising from natural and anthropogenic sources. Due to long history of industry, approximately three million sites are estimated to have been contaminated in Europe (Nicholson et al., 2003; European Commission, 2013; Environmental Agency, 2004; SEPA, 2006). The presence of soil and water contamination in other parts of the world has also been established (USEPA, 2001; Li et al., 2006).

Heavy metals such as Zn are released into the environment from both natural and anthropogenic processes. Zinc is naturally present in atmospheric emissions from volcanoes, the weathering of rocks, mineral dusts, and forest fires (Oves et al., 2012; Alloway, 1995). Industrialization and urbanization have amplified the anthropogenic sources of Zn to the environment through the exploitation of mines and smelters, sewage sludges, combustion of fossil fuel, metallurgical industries,

intensive agricultural practices and pesticides, military operations, paints, gas works, battery manufacturers and electronics (Alloway, 1995; Vangronsveld and Cunningham, 1998; Gadd, 2010). Each source of contamination has its own detrimental effects to the entire ecosystem as most metals do not undergo microbial or chemical degradation. Instead, they are being transformed from one oxidation state to another (Lone et al., 2008) so that total metal concentration in soil persists for a long time after metal release in the environment. The mean natural concentration of zinc in the Earth's crust is 70 mg kg^{-1} (dry weight), the total Zn concentration in soil ranges from 10 to 300 mg kg^{-1} with an average of 55 mg kg^{-1} (Alloway, 2008). Zn has been identified as a metal that poses a potential threat to the entire ecosystem (Gadd, 2010). Consequently, some countries (e.g. United Kingdom) have set out guidelines to regulate heavy metal concentrations in soil amendments including Zn and have defined the maximum allowable heavy metals concentration in soils (Nicholson et al., 2003) as seen in Table 2.1.

Table 2.1. Maximum concentration of heavy metals in sludge amended soils and maximum annual rates of addition by the UK sludge (use in Agriculture) Regulations (UK statutory Instrument, 1989; DoE, 1996).

Metal	**maximum permissible concentration in soil (mg/kg dry soil)**				**Maximum permissible annual average rate of addition over 10 yr (Kg ha^{-1} yr^{-1})**
	pH 5.0<5.5	**pH 5.5<6.0**	**pH 6.0-7.0**	**pH >7.0**	
Zn	200	250 (200) †	300 (200) †	450 (300) †	15.0
Cu	80	100	135	200	7.5
Ni	50	60	75	110	3.0
	pH 5.0 and above				
Cd	3				0.15
Pb	300				15.0
Hg	1				0.1
Cr	400				15.0
As	50				0.7

† Values in parentheses are UK advisory limits (Ministry of Agriculture, Fisheries and Food, 1993).

2.3 Metal bioavailability

The term bioavailability has several meanings across different disciplines. With respect to contaminated soil, bioavailability can be defined as the maximum amount or the total quantity of an element present in a specific partition of the environment, that is either available or more available for uptake and accumulation by an organism within a given time span (Peijnenburg and Jager, 2003; Peijnenburg et al., 2007). The bioaccessible fraction is the fraction of metal that is in contact with the organism surface and is available for absorption or adsorption by the organism. Both terms bioaccessibility and bioavailability are complex factors that regulate whether or not an adverse effect will occur when plants are exposed to contaminants (Peijnenburg and Jager, 2003). The concept of bioavailability is complex since it is soil, species, and chemical specific and dynamic due to ongoing processes of bioaccumulation, distribution, metabolism and elimination (Ianni et al., 2010). Although, some assessments have assumed that metals are totally bioavailable or bioaccessible from soil, only a small fraction of metal is bioavailable (Violante et al., 2010). Several studies have reported that the relative bioavailability of metals to organisms tends to decrease with time, following addition of metals to soil (Gigliotti et al., 1996; Zheljazkov et al., 2004; Petruzzelli et al., 2012). Metals in soil are partitioned in five geochemical forms: exchangeable, bound to organic matter, bound to carbonate phase, bound to manganese oxides and residual metal (Oves et al., 2012). Metals bound to organic matter or adsorbed to solids are no longer free and thus less bioavailable. Metals found in different fractions or forms vary in mobility and bioavailability, which is strongly influenced by the chemical speciation (Violante et al., 2010). The mobility, bioavailability and toxicity of a metal in soil depends on its concentration in soil solution, its reaction with other soluble species and soil ability to release metal from the solid phase (Krishnamurti et al., 2007; Violante et al., 2010). The availability of Zn in soil is controlled by the factors that affect the amount of Zn in soil solution. These soil properties include total zinc content, soil pH, cation exchange capacity, exchangeable cation, organic matter content, mineralogy and clay content (Kretzschmar and Voegelin, 2001; Zhao et al.,

2012). The soil processes affecting Zn availability include adsorption, desorption, precipitation, dissolution, complexation, amongst others (Ma and Uren, 2006; Kim et al., 2015) and are described in detail in the next section.

2.3.1 Soil properties and processes affecting metal bioavailability in soil

Soil pH

Soil pH is defined as the negative logarithm of the hydrogen ion concentration (Alamgir, 2016). Soil pH is a vital factor that influences contaminants and biological processes in soil. The biological processes governed by pH are solubility, precipitation and dissolution, sorption, desorption and microbial activities (McLean and Bledsoe, 1992). A low pH tends to lead to a decrease in sorption and an increase in bioavailability and mobility of most metals (Rieuwert, 2007). Zn bioavailability is strongly affected by soil pH (Turner, 1994; McGrath et al., 1988). In Zn contaminated soils as pH decreases, the solubility and extraction of Zn from soil increases (Parker et al., 1990; Saeed and Fox, 1997; Adriano, 2001). Twining et al. (2004) reported that between pH 4.4 and 7.3 the distribution coefficient (K_d) for Zn increased from 40 to 3000 mL / g in laterite soil in Australia. Zn, Cd and Ni were mobilised under low pH in soils from a mining area in Togo (Gnandi and Tobschall, 2002). Conversely, at high soil pH, Zn is more strongly absorbed on to the surface of silicate clays and oxides and hence the availability of Zn to plants is reduced (Lindsay, 1978; 1981; Jeffery and Uren, 1983). In calcareous and alkaline soils, precipitation of $ZnCO_3$, calcium zincate compounds or $Zn(OH)_2$ may reduce Zn availability for plant uptake (Saeed and Fox, 1997).

Cation exchange capacity

The cation exchange capacity (CEC) of a soil is the capacity to hold cations on (positively charged ions such as $Mg^{2+,}$ Ca^{2+}, Na^{+}, K^{+}, H^{+}, Al^{3+}, Mn^{2+}, Zn^{2+}) on negatively charged sites (Pulford, 2007). CEC are vital to soil because of their fixed or permanently charged sites which are not affected by changes in soil pH and as such cations held by soils are replaced by other cations (McLean and Bledsoe, 1992) this implies that they are exchangeable. The CEC of solids is dependent on their mineralogy and organic matter content (Bell, 1993). Both clay particles and organic matter particles are negatively charged constituents of soil which may also contain pH dependent charged surface. The higher the soil organic matter and clay content, the higher the CEC. Soils with negative charge have high cation exchange capacity and low cation mobility (NFESC, 2000). The higher the cation exchange capacity of soil, the greater the sorption and immobilization of the metals.

Organic matter content

Soil organic matter is made up of living organisms, soluble biochemical (organic acid, polysaccharides, amino acids, proteins amongst others), and humic matter (Covelo et al., 2008). The origin of soil organic matter greatly influences its physicochemical properties (solubility, surface charge sorption). Organic matter has a high specific area (800 – 900 m^2/g) and a CEC that ranges from 150 to 300 cmol / kg (Sparks, 2003) and is thus considered to be an important adsorbent of heavy metals. As the amount of organic matter in soil increases, the tendency for forming stable metal- organic matter complexes increases (Reichman, 2002). Zinc forms stable complexes with organic ligands such as insoluble organic colloids (humin) and dissolved organic carbon (fluvic, humic, amino and carboxylic acids) (Cavallaro and McBride, 1984). Plants are unable to absorb the large metal– organic complexes, resulting in reduced metal availability. Zinc becomes less phytotoxic when

complexed with organic matter (Jones, 1998) thus improving plant growth and Zn tolerance.

Clay and hydrous oxides

Clay minerals are products of rock weathering (Nesbitt, 1992), have diameter less than 2 µm (Yu et al., 2015) and are important soil constituents. Clay minerals possess a high surface charge density as a combination of their small size and high surface charge resulting in an active surface for cation adsorption (Alloway, 1995; Pulford, 2007). Clays and hydrous oxides (of Al, Fe, and Mn) act as natural absorbing agents of contaminants by taking up anions and cations by adsorption and / or ion exchange, thereby limiting their availability in soil solution (Jain and Ram, 1997; Bhattacharyya and Gupta, 2008). Increasing clay and hydrous oxide content in soils provides more sites for adsorption of metals thus reducing metal bioavailability (Qiao and Ho, 1996; Reichman, 2002).

Redox conditions

Redox is a measure of electron availability as electrons are transferred between oxidized and reduced species (Gambrell et al., 1991; Rieuwets et al., 2003). The redox conditions of a soil are affected by a number of factors, including compaction, waterlogging and organic matter content. Soils rich in organic matter and prone to waterlogging have low redox potentials while high redox potential occurs in dry, well aerated soil (Evans, 1989). Redox reactions in soil are controlled by metal species in the soil solution and solubility of metals in solution (Reichman, 2002). Some studies reported that Zn availability increases in poorly drained, waterlogged soil (Ng and Bloomfield, 1962; Chuan et al., 1996) whilst other authors concluded that waterlogged soil and low redox conditions do not increase metal solubility (Xiong and Lu, 1993; Bjerre and Shierup, 1985) due to the formation of metal sulphides and thus decrease metal bioavailability (Rieuwerts et al., 1998).

Aging of metals in soil

Another factor affecting metal bioavailability is chemical aging in soil (Alexander, 2000). Metal aging in soil is the movement of metal from the surface of soil particles into less accessible sites with time (Reid et al., 2000). The bioavailability of metals can decrease with time as metals in soil undergo processes that inhibit their desorption from the soil solid phase to soil pore water (Gaw, 2009), such as binding with Fe and Al oxides and minerals of low reactivity (Ma and Uren, 2006). As metals age in soil their bioavailability decreases (Pignatello and Xing, 1996; Turkall et al., 2010).

Adsorption and desorption

Adsorption is the accumulation of substances or material at the interface between the solid phase and solution (Sparks, 2003). Adsorption occurs when dissolved metals are attached to surfaces of soil particulate matter (clay, organic matter, iron, manganese and aluminium oxide minerals) (Kabata-Pendias and Pendias, 1984; Elder, 1989). The absorbing surface is said to be the "absorbent" (solid particles) and the material adsorbed at the surface is called the adsorbate (Spark, 2003). Often the generic term sorption is used to encompass all phenomena, including precipitation and adsorption, at the solid –solution boundary. Sorption and desorption reactions are important controls on metal bioavailability in soil. Sorption by soil is a multistep process involving an initial rapid sorption followed by slow sorption, probably by diffusion into pores of inner soil surfaces, due to the presence of surface sites of different reactivity and site preference (Violante et al., 2008). Factors affecting trace metal sorption include pH, and the content of humic substances, phyllosilicates and variable-charge minerals (hydroxides and oxyhydroxides of Al, Fe, Mn, and aluminosilicates) which differ greatly in their sorption capacity, binding energies of their sorption sites and in their cation and anion exchange capacities (Bourg, 1988; Sparks, 2003; Violante et al., 2005). pH is considered the most important environmental factor since it affects solubility and

speciation of metal in soil and soil solution (Harter, 1983). At low soil pH values, adsorption site becomes positively charged due to adsorption of protons. As pH increases, these sites become neutral and eventually negatively charged. The negatively charged adsorption sites are compensated by equivalent amounts of positive charges. Elliot et al. (1986) reported that soil adsorption of heavy metals varied directly with soil pH, with metal ions likely to exist in soil solution at low pH (Zeng et al., 2011).

The processes of Zn adsorption in soil have been reviewed (Pickering, 1980; Shuma, 1980). Soil pH and contents of clay minerals, hydrous oxides and organic matter are the most important factors affecting Zn adsorption in soil (Abd-Elfattah and Wada, 1981; Barrow, 1993; Barrow and Whelan, 1998**).**

Desorption is the reverse of adsorption, involving the electrostatic release of hydrogen bonded or electrostatically attracted soluble organic or inorganic cations from negative sites on soil colloids into the bulk solution (Brady and Weil, 2002; Bradl et al., 2005). Desorption process is expected to occur at a much slower rate compared to adsorption (Violante et al., 2008).

Metal particle size and resulting total surface area can influence bioavailability (Deletic and Orr, 2005) with bioavailability increasing as particle size particle decreases (Rieuwert, 1998). Small particles with large surface area tend to bind more contaminants than large particles (Xu et al., 2014). The concentration of metal in soil increases as particles decreases (Chaney et al., 1989; Xu et al., 2014).

Dissolution and precipitation.

Dissolution and precipitation are chemical processes that determine the availability of inorganic mineral components of soil as dissolved metals are more mobile and available than precipitates.

Precipitation occurs when the solubility product constant for a reaction between a metal ion and ligand in the system is exceeded (Peijnenburgh and Jager, 2003). Metal are immobilized as precipitates in the presence of anions such as sulphate, phosphate, carbonate when the soil pH and metal concentration are high (Adriano, 2001). Compared to sorption and desorption, precipitation of metal is a slow process and is less likely to occur due to low metal concentration (Sparks, 2003). Brummer et al. (1983) reported that very high concentration of Zn can be adsorbed in soil before precipitation occurs. Metals may also be precipitated as metal oxides and hydroxides (Rieuwert at al., 1998) which are the most common forms of metal precipitates. Precipitation as sulphides also occurs and is an effective process for precipitation of highly toxic heavy metal (Park et al., 2010).

Dissolution, the reverse of precipitation, involves the separation of solids into individual soluble components (Bradl et al., 2005). The extent to which metal species occur in a particular soil and their solubility determines their relative bioavailability (Rieuwert et al., 1998). Dissolution and Precipitation processes are dependent on pH and metal ion present in solution (Rieuwert at al., 1998).

Complexation

Metal complexation involves a centrally located metal ion in solution being attached to one or more inorganic or organic ligands (Rieuwert, 1998). Complexation may occur as chelation where the complex forming ligands form two or more coordination bonds with the metal ion (Lindsay, 1979). These ligands can form

soluble complexes with trace metals, thus preventing them from being sorbed onto solid surfaces and increasing their availability. For example, soluble Zn complexes are formed by removing free Zn from solution thus increasing Zn dissolution at the solid –phase (Essington, 2004). Natural complexing ligands include citric acid, oxalic acid and fulvic and humic acid fractions (Violante et al., 2010) and synthetic complexing ligands include ethylenediaminetetraacetic acid (EDTA),

ethylenediaminedisuccinic acid (EDDS) and diethylenetriamine pentaacetic acid (DTPA) (Kolodynska, 2013).

Chemical speciation

Metals occur in the soil environment in both the aqueous phase and solid phase. In solution, metals exist as complexes associated with organic and inorganic ligands or as the free ion (Rieuwerts et al., 1998; Violante et al., 2010). In the solid phase, metal ions can be either retained on organic and inorganic soil components (ion exchange or surface complexation) or can exist as minerals or co-precipitated with other metals in soil (Violante et al., 2008). Since the form in which a metal occurs influences its bioavailability, the speciation of Zn is described in detail below.

(i) Zinc

Zinc is a metallic chemical element (with atomic symbol Zn), has an atomic number 30 and is found in five isotopic forms: ^{64} Zn, ^{66}Zn, ^{67}Zn, ^{68} Zn and ^{70}Zn. Zinc exists in the environment as Zn^{2+} with an electron configuration of [Ar] $4s^2$ $3d^{10}$ making it a member of group 12 of the periodic table. Because the d-subshell is full of electrons it does not function as a transition metal. The localised d- electrons of Zn play a significant role in controlling its chemical and physical properties (such as the melting points) (Da Silva and Williams, 1991; Nasch et al., 1999). Zinc occurs in the environment in the +1 and +2 oxidation state (Lindsay, 1979; Brady et al., 1983), with Zn^{2+} predominantly involved in biological or chemical reactions. Zinc in soil solution may occur as Zn^{2+} in association with a range of organic or inorganic

components and minerals, (Fotovat and Naidu, 1998; Brady and Weil, 2002; Essington, 2004). Zinc is partitioned into different chemical pools in soil (inorder of decreasing availability of plant uptake): in soil solution, on exchange sites of reactive soil components, precipitated and occluded with oxides, oxyhydroxides, carbonate and phosphate, in complexes with organic matter, and finally in primary and secondary minerals (Alloway, 1995).

(ii)Chemical forms of Zn in soil

Zn occurs in the mineral component of soils including as: sulphides (sphalerite, wurtzite); sulphate (zincosite, goslarite); oxides (zincite, franklinite, gahnite); carbonates (smithsonite, hydrozincite); silicates (willemite, hemimorphite); and phosphates (hopeite) (Barak and Helmeke, 1993; Alloway and Ayres, 1997). Aqueous Zn is present both in acidic to highly basic soil solutions in various hydrolysed forms such as Zn $(H_2O)_6^{2+}$, Zn $(OH)(H_2O)_5^{+}$, $Zn(OH)(H_2O)_4^{0}$, $Zn(OH)_3(H_2O)_3^{-}$, $Zn(OH)_4(H_2O)_2^{2-}$ (Barak and Helmeke, 1993). Zinc may form complexes with ligands of humic substances such as fulvic acid, humic acid and dissolved organic (humic) carbon. Although Zn forms complexes with organic acids potentially found in soils (citric, malic and oxalic, amongst others), these ligands are rarely analysed in soil solution (Barak and Helmeke, 1993).

2.4 Determination of metal speciation

The speciation of metal can be determined by different techniques, which are discussed below.

2.4.1 Sequential extraction

Sequential extraction is an approach for the fractionation of trace metal content in soils and sediments, providing information on the various forms of the chemical element (Mossop and Davidson, 2003; Sepahvand and Forghani, 2012). The

quantification of chemical forms is based on the use of chemical reagents selected to react with components of the matrix and the release of associated trace metals (Mossop and Davidson, 2003). Several sequential extraction methods have been proposed for defining individual fractions of a given elements. For example, the first sequential extraction procedure was described by McLaren and Crawford (McLaren and Crawford, 1973) and modified by Tessier et al. (1979) who developed a five stage extraction (exchangeable, carbonate, Fe, Mn and Al oxide, organic and residual fractions) to evaluate the fractions of Cd, Cu, Fe, Pb, Mg, Ni and Zn in river

sediments. Sposito et al. (1982) proposed a four stage sequential extraction scheme on the fractionation of Pb, Cd, Zn, Cu and Ni in soils amended with sewage sludge. Standards, measurement and testing (SMT) formerly known as community bureau of reference (BCR) proposed a three stage sequential extraction scheme (acid soluble, reducible and oxidizable fractions) which is internationally accepted (Rauret et al., 1999; Fuentes et al., 2004; Tlustos et al., 2005; Cheng et al., 2011; Ashraf et al., 2012; He et al., 2013). This method has been applied to investigate the distribution and speciation of metals in different environmental media such as soil (Mossop and Davidson, 2003; Fernadez et al., 2004; Nemati et al., 2011), waste material (Bruder-Hubscher et al., 2002) and sediment (Stephens et al., 2001; Yuan et al., 2004; Cuong and Obbard, 2006). Wang et al. (2010) investigated the distribution and speciation of Cu, Cd, Pb, Mn and Fe in sediments and reported that 39% - 61% of Cd was found in exchangeable fraction, indicating that Cd in the sediment posed a high risk. Although sequential extraction has been effectively used, some researchers report difficulty with this technique due to lack of fraction selectivity, redistribution of analytes between phases (Pueyo et al., 2001) and operational problems (Martins et al., 1987).

2.4.2 Transmission electron microscopy (TEM)

Transmission electron microscopy is a microscopy technique that uses energetic electrons to provide compositional, morphological information on both organic and inorganic material (Smith et al., 2012) at the nanometre scale (Howe, 2012). TEM coupled with EDS (energy dispersive spectroscopy), provides information on the size, morphology, crystallinity and elemental composition of single particles as well as their physical and spatial association (Webb et al., 2000). TEM-EDS has been used to assess chemical speciation in lake sediments, mineral association in aquifer water and other geochemistry studies (Webb et al., 2000). The limitation of this method is that samples have to be studied in their wet state to ensure structural preservation and avoid shrinkage and aggregation of particles (Webb et al., 2000; Kourkoutis et al., 2012).

2.4.3 X-ray absorption spectroscopy (XAS)

X –ray absorption spectroscopy is a method for analysing the chemical environment of an element in an unknown material (Kawai, 2000). Recently, XAS has been applied to investigate metal coordination and interactions within biosystems such as soil and plants (Gardea-Torresdey et al., 2005), providing detailed information on their atomic geometry, the coordination environment of metals absorbed by plants, and bioreduction of metals linked to phytoremediation (Saraswat and Rai, 2011). Synchrotron – based methods such as XANES (x-ray absorption near edge surface) and EXAFS (extended x-ray absorption fine structure) are two components of XAS which have been used for understanding the chemical composition and distribution of elements in environmental samples (Donner et al., 2013). Whilst XANES spectroscopy provides useful information on chemical oxidation state and coordination geometry of elements in complexes (Parsons et al., 2002), EXAFS provides detailed information on the co-ordination geometry of the investigated atoms in the sample (Gardea-Torresdey et al., 2005). Castillo- Michel et al. (2012) used this method to investigate As (arsenic) speciation in soils and plants (*Prosopis juliflora – velutina)*. The speciation and distribution of Zn in mesquite plants grown in ZnO nanoparticle media was studied using XANES (Hernandez-Viezcas., 2011). Salt et al. (1999) reported that Zn was coordinated with histidine in the roots of *Thalaspi caerulescence*, using EXAF. Zn was found to be coordinated with Zn malate, Zn citrate and Zn phosphate in the roots of *Arabidopsis halleri* (Sarret et al., 2002). Straczek et al. (2008) reported Zn was predominantly coordinated with COOH / OH groups (40 -80 % of total root Zn) in the cell wall of tobacco plant roots. Adediran et al. (2015) also observed Zn coordination in *Brassica Juncea (L.) Czern* through XANES and reported Zn was complexed predominantly with Zn oxalate and Zn sulphate when roots were not inoculated with bacteria, where as in roots inoculated with *Pseudomonas brassicacearum* with *Rhizobium trifolii,* Zn was found to be coordinated with Zn polygalacturonic acid. These studies generally suggested that coordination of Zn with inorganic and organic ligands is an important mechanism for Zn tolerance and detoxification in plants.

XAS has proven to be useful in understanding metal speciation in environmental samples. However, XAS provides weighted average structural information and does not ascertain the absolute sum of all the species present in a sample (Carey et al., 2012). It also has limited sensitivity and requires synchrotron x-ray source (Templeton and Knowles, 2009; Nearing et al., 2014).

2.5 Contaminated land exposure assessment and its importance in metal availability

Contaminated environment is a world legacy of industrialisation, often requiring huge investments to remediate (Riding et al., 2013). Metals, metalloids, nanoparticles amongst others are a major component of environmental contamination. These contaminants are persistent and toxic thus studying their sources, transference and potential human health effects in the environment is of great significance (Goix et al., 2016). According to Hough et al. (2004) under part IIA of the Environmental Protection Act 1990, Land is contaminated only if the current or intended use of a site has the potential to cause an unacceptable health risk to human or to the environment. This means that a quantitative assessment of the hazard and risk of metal pollutants is placed in the context of the future use and receptor for that site (Alhadrami et al., 2016). However, when considering metal impact on receptors through several exposure pathways, a measure of the total metal content may be inadequate (Ruby et al., 1996; Alhadrami et al., 2016).

Biological availability that simulate receptor's exposure to soil provide a more realistic approach and estimate of human exposure to hazard (Ruby et al., 1996; Alhadrami et al., 2016). Environmental hazard is described in terms of metal bioavailability and mobility in soils. The mobility of metal describes metal partitioning between soil solids and solution (Ianni et al., 2014). Metal bioavailability or bioaccessibility is defined as the total metal fraction that is accessible (or soluble) and available for absorption in the target organ (Goix et al., 2016). Therefore, measuring metal bioavailability can be used to refine exposure assessment and risk characterization (Alhadrami et al., 2016; Goix et al., 2016;

Guney and Zagury, 2016). Information on contaminant bioavailability has been widely recognised among researchers, legistors and regulators (Latawiec et al., 2010; Zhang et al., 2013; Guney and Zagury, 2016).

A risk assessment tool can be any methodology, model or software package designed to qualify or quantify the risk posed by a contaminant in evaluating a source-pathway-receptor linkage (kumar, 2015). Risk assessment tools that have been developed to support the approach to contaminated environment is discussed below

CLEA- Contaminated Land Exposure Assessment

CLEA is sponsored and developed by the UK, Environmental Protection Agency and is one of the few models that considers background exposure (Chen, 2010). CLEA is an exposure model that uses generic assumptions about the fate and transport of chemicals in the environment and a conceptual model for site conditions and human behaviour to estimate child and adult exposure to soil contaminants for those potentially living, working, and /or playing on contaminated sites over long periods (Environmental Agency, 2009a). There are 10 pathways of human exposure to soil contaminants and the model can include further concentration measurements, such as concentration levels measured in soil, air and plants (Environmental Agency, 2009b). CLEA compares directly the estimated average daily

exposure with the relevant health critical value from representative site concentrations in soil, air and plants using ratio mode. CLEA derives site-specific assessment criteria by combining standard assumptions with further site-specific information collected in order to refine the risk assessment (Environmental Agency, 2009b). Generic soil guidance (SDVs) are of significance in CLEA (Defra and Environmental Agency, 2002). Where a soil exceeds the SGV, it is recommended that a risk assessment or remediation measure be conducted for the site in question (Defra and Environmental Agency, 2002). Additionally, exceeding of an SGV indicates that further risk management action should be undertaken (Rimmer et al., 2006; Islam et al., 2007).

2.6 Zn function in plants

Along with several metal contaminants, zinc (Zn) is an essential micronutrient required for plant growth and development (Remeo et al., 2014). Zn is a major component of about 300 metallo-enzymes that maintain functional, structural and regulatory systems in plants (McCall et al., 2000). Zn acts as a catalytic or structural cofactor of several enzymes such as anhydrase, dehydrogenase, oxidase and peroxidase (Kabata-Pendias and Pendias, 2001). Zn is also involved in the metabolism of carbohydrates and proteins, synthesis of tryptophan and indole acetic acid (Cakmak et al., 1989) and production of auxins in plant species (Marschner, 1986). The regulation and maintenance of the gene expression required for the tolerance of environmental stresses in plants are Zn dependent (Cakmak, 2000). Zn affects the capacity of water uptake and transport in plants and alleviates the effects of heat and salt stress (Kasim, 2007; Disante et al., 2010; Peck et al., 2010). Zn is required for the maintenance of cellular membranes to preserve the structural orientation of macromolecules and ion transport systems (Marschner, 1995). Zn interaction with phospholipid and sulfhydryl contributes to the maintenance of membranes group (Cakmak, 2000; Kabata-Pendias and Pendias, 2001; Alloway, 2004; Disante et al., 2010). Norvell and Welch (1993) reported that Zn prevented the oxidation of sulfhydryl groups to disulfides in the root membrane.

2.6.1 Zinc toxicity and deficiency in plants

It was reported by Katyal and Randhawa (1983) that the growth and yield of plants is strongly related to Zn concentrations in the tissues of plants. Zn concentration in plants varies with different soil, plant species, and climatic factors which influence deficiency or toxicity in plants. Zn concentration (mg Zn/kg) in plants can be generally classified as: “deficient” if < 10 to 20, “sufficient” or normal if between 25 and 150, and “excessive” or toxic if > 400 (Kabata-Pendias and Pendias, 1984). Zn toxicity to plants depends on pH of soil which controls the concentration of Zn in solution since free Zn^{2+} in solution is highly toxic to plants (Zhao et al., 2012).

Accumulation of excess zinc in soil is an environmental issue due to its toxic effect on plants. The effect of elevated zinc concentrations on plants has been investigated in many studies. High levels of Zn in plant lead to reduced yields and stunted growth, Fe-deficiency-induced chlorosis through reduction in chlorophyll synthesis and chloroplast degradation (Mirshekali et al., 2012). Stunt growth and decrease in mitotic activity of roots (Jain et al., 2010), inhibition of cell elongation and division, (Arduini et al., 1994; Cakmak, 2000; Khudsar et al., 2004), interferences with other nutrients (P, Mg and Mn) uptake (Foy et al., 1978; Chaney, 1993; Kaya et al., 2000) and inhibition of enzyme activities (Khudsar et al., 2004). Excess zinc can also hinder stomatal conductance (Khudsar et al., 2004; Dhir et al., 2008; Sagardoy et al., 2010) as well as inactivate enzymes involved in CO_2 fixation (Khudsar et al., 2004), reduce seed sprouting and seedling stunting, chlorosis, necrosis (Foy et al., 1978; Wong and Bradshaw, 1982). Furthermore, exposure to increased Zn concentrations has been shown to enhance production of reactive oxygen species (ROS) in plant cells, thus causing cells to be exposed to oxidative stress resulting in ion leakage, lipid peroxidation, membrane damage and biological macromolecule deterioration (Wintz et al., 2003; Feigl et al., 2015). Beyond direct effects in the plants, soil enzymes, microbes and nitrogen fixation are inhibited, in Zn contaminated soil (Doelman and Haanstra, 1984; Baath, 1992; Mhatre and Pankhurst, 1997).

2.7 Nanoparticles

Particles that have at least one dimension between 1 and 100 nm are said to be nanoparticles (Glover et al., 2011; Pan and Xing, 2012). NPs have a larger surface area to volume ratio than their bulk counterparts allowing for greater reactivity, and possess unique physicochemical properties that makes them exhibit different characteristics to those displayed by "bulk" materials of identical chemical composition (MacCormack and Goss, 2008; Nie et al., 2015).

2.7.1 Characteristics and applications

Nanoparticles can be grouped based on their morphology (sphericity, flatness and aspect ratio); dimensionality (1D, 2D and 3D); agglomeration and uniformity (nanoparticles can exist as colloids, in suspension, dispersed aerosols or in an agglomerated state); composition (nanoparticles can be composed of one material or several materials (Nowack and Bucheli, 2007; Remedios et al., 2012) and source.

Naturally, nanoparticles are abundant in all environments, both aquatic and terrestrial, as they are produced by many natural processes including volcanic eruptions, chemical and physical weathering of rocks, biological processes and precipitation reaction (Hochella et al., 2015; Sharma et al., 2015). Engineered or manufactured nanoparticles are also formed as products from human activities such as combustion of coal and fuel oil, ore refining and smelting (Plathe et al., 2013). Currently, nanoparticles are of great scientific interest as they have many potential applications in technology, commercial and industrial sectors (Mitrano et al., 2015; Soenen et al., 2015; Stark et al., 2015). This is influenced by their nanosize, optical, mechanical and electrical properties which have resulted in a range of nanotechnology material products, including carbonaceous nanomaterials and semiconductor, metals, metal oxides and nanopolymers (Mitrano et al., 2015; Soenen et al., 2015; Stark et al., 2015). Metal oxides are among the most extensively used nanoparticles. For example, due to their electrical and optical properties (Heinlaan et al., 2008). Zinc oxide nanoparticles are widely used in UV absorbing material, gas sensors and coatings for solar cells (Hernandez-Viezcas et al., 2011). Other nanoparticles such as ZnS nanoparticles are widely used in

photoluminescence, and optoelectronics (e.g in reflector, light emitting diode, and window material) (Rahdar et al., 2012).

2.7.2 Nanoparticles in the environment

Apart from their useful industrial applications, nanoparticles (NPs) have the potential to enter the environment and contaminate land, soil and sea and fresh and saline waterbodies (Klaine et al., 2008; Peralta-Videa et al., 2011; Zhu et al., 2016). NPs may be introduced intentionally or unintentionally into the environment through several sources such as accidental spillage during production or transportation and diffuse release which is associated with production facilities and leaching from solid waste (Mueller and Nowack, 2008; Keller et al., 2013). Keller et al., 2013 identified the release of ten common NPs (ZnO, carbon nanotube (CNT), silica (SiO2), titanium dioxide, alumina, iron and iron oxide, nanoclay, cerium oxide, silver, copper and copper oxide) in the environment. Majority of these released NPs ended up in landfill, with most NPs emitted to soil and water (Muller and Nowack, 2008; Keller et al., 2013). Specifically, NPs are directly released into soil through the use of nanofertilizers, nanopesticides, agrofilms materials (Gladkova and Terekhova, 2013). Other deliberate sources of NPs released into the environment includes, the use of NPs in water and soil remediation technologies (Barnes et al., 2010). Land application of organic amendment (including biosolids, sewage sludge and manure) represents a significant environmental exposure for NPs (Yang et al. 2014; Santiago-Martin et al., 2016). The presence of NPs in the environment may have an adverse effect on man (via inhalation, digestion or exposure to air, soil and water) and the entire ecosystem (Klaine et al., 2008; Papp et al., 2008). The potential release of NPs in the environment has not been fully monitored, thus evaluating the associated risk is difficult (klaine et al., 2008; Maurer-Jones et al., 2013; Hegde et al., 2016). Nevertheless, monitoring NPs in the environment remains a major challenge as development of techniques for detection, identification and quantification of NPs is ongoing (ENRHES, 2010; Bakshi et al., 2015).

2.7.3 Fate of nanoparticles in soil.

Soil represents a natural medium with which nanoparticles may interact (Pan and Xing, 2012). On-going studies of the behaviour of nanoparticles in the aqueous media might be of relevance to soils as the nanoparticle behaviour in the aqueous phase of soil may be of great importance for their bioavailability and transport (Tourinho et al., 2012). Characteristics that influence the behaviour of nanoparticles in the environment include: particle state (e.g. size, structure, surface area and surface charge), chemistry (e.g. ionic strength, natural organic matter content, pH, ionic composition (Petosa et al., 2010) aggregation, agglomeration, dissolution and dispersion (Pan and Xing, 2012). Most NPs will also under go significant transformation processes which include oxidation, sulphidation which will result in less reactive NPs that are less likely to dissolve and be less toxic (Lv et al., 2012; Ma et al., 2013; Garner and Keller, 2014). These characteristics of nanoparticle also influence the degree to which they undergo change that controls their fate, behaviour and toxicity in the environment (Tourinho et al., 2012; Cornelis et al., 2014; Garner and Keller, 2014).

2.7.4 Implication of nanoparticles for toxicity

Increases in production and usage of nanomaterials will result in increased exposure to living organisms (Wang et al., 2016). The physicochemical properties of nanoparticles such as sizes, chemical composition and sturcutures, and high surface area to volume ratio of nanoparticles will enhance toxicity resulting in potential damage to humans, microorganisms and plants (Simon and Richaume, 2015; Sajid et al., 2015; Ebbs et al., 2016; Hegde et al., 2016). Nanoparticles may potentially have an adverse effect on the environment due to their abundance and long residence time (Quigg et al., 2013). There are several mechanisms by which nanoparticles will have toxic effects on living cells. Due to their redox activity, nanoparticles can damage cells as a result of the production of reactive oxygen species (ROS) which might lead to oxidative stress in cell (Klaine et al., 2008; Manke et al., 2013; Sarka et al., 2014; Khanna et al., 2015). ROS induction is due to nanoparticles-cell interaction or the presence of pro-oxidant functional groups on

their reactive sites, which is mostly, considered the primary cause of nanotoxicity (Khanna et al., 2015). Nanoparticles damage the membrane integrity, which could lead to increases in membrane permeability causing cells to be more susceptible to osmotic stress and also hindering nutrient uptake (Friehs et al., 2016). ZnO NPs increases the leakage of the cell membrane (Yang et al., 2009), Ag NPs directly interacted with membranes of Escherichia coli and increased their permeability (Duran et al., 2016). NPs may also interrupt energy transport processes due to retention of electrons (Pan and Xing, 2012). Several studies have shown that nanosized particles can be taken up by mammalian cell types (Osman et al., 2010; Roszak et al., 2016), and may negatively affect soil bacteria (Kim et al., 2013). Nano - TiO_2 and nano- ZnO showed toxic effects as they reduced the microbial biomass, and resulted in changes in the composition and diversity of the bacterial community (Ge et al., 2011), and changes in soil enzyme activities such as catalase, peroxidase and protease (Du et al., 2011).

NPs also interact with plants, causing morphological and physiological changes depending on the NPs properties (Siddiqui et al., 2015). Both negative and positive effects of NPs on plants have been reported, depending on the nanoparticles concentration, plant species and the physical and chemical properties of NPs (Ma et al., 2010).

The effect of NPs on photosynthesis and gas exchange in food crops have been reported (Das et al., 2015; Rao et al., 2014). NPs caused a significant decrease in chlorophyll content in Indian mustard, pea and soybean (Nair et al., 2013; Rao et al., 2014; Mukherjee et al., 2014). It has been reported that NPs caused oxidative stress in plants by producing reactive oxygen species (ROS) in many plants (Rajeshwari et al., 2015). For example, CuO and ZnO NPs caused oxidative stress in wheat by increased lipid peroxidation and oxidized gluthathione in roots (Dimpka et al., 2012). NiO NPs caused a significant (122%) increase in intracellular ROS production in tomato roots (Faisal et al., 2013). NPs have been shown to affect mineral nutritional status in plant (Li et al., 2014). For example, Ag NPs decreased mineral elements (B, Zn, Ca, P, Fe, Cu, Mn, Mg and Na) in tomato seedling (Shams et al.,

2013). NPs have caused genotoxicity in plants by modification of plant gene expression (Rizwan et al., 2016; Nair et al., 2015). Shaymurat et al. (2012) reported that ZnO NPs (10 - 50 mg L^{-1}) induced mitotic aberration and decreased the mitosis index in *Allium sativum* (garlic). Similar genotoxcity effect of ZnO NPs on onion root tips have been reported (Raskar and Laware, 2014; Taranath et al., 2015). NPs reduced crop yield and nutritional quality of crops. Zhao et al. (2014) reported that CeO_2 and ZnO NPs (400 and 800 mg kg^{-1}) altered the quality of protein, mineral nutrients and carbohydrate of cucumber. Other negative effects of NPs on plant growth and morphology have also been reported (Mahajan et al., 2011; Kim et al., 2012; Kouhi et al., 2015). For example, ZnO NPs (500 and 750 mg kg^{-1}) reduced the shoot and root biomass of alfalfa by 80% and 25% (Bandyopadhyay et al., 2015).

Conversely, some studies reported positive effects of nanomaterials on plants for example; Ag NPs increased ascorbate and chlorophyll in the leaves of *Asparagus officinalis* (Asparagus) (An et al., 2008), TiO_2 decreased malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and electrolyte leakage in chickpea (mohammadi et al., 2014), Silica NPs increased seed germination, biomass, shoot and root length and chlorophyll content in maize under field condition (Suriyaprabha et al., 2012). ZnO NPs promoted the root growth of soybean (Oberdorster, 2010). Other positive effects on plant growth have also been reported (Wang et al., 2013; Lee et al., 2010; Morales et al., 2013).

2.7.5 Mechanism of Phytotoxicity

The phytotoxicity of NPs has been well investigated but the mechanism of phytotoxicity is still unclear since nanomaterials and organisms interact in many ways (Diets and Herth, 2011) including: (1) metal ion release by NPs in solution elicit a chemical effect; (2) hard spherical NPs produce mechanical effects; (3) the NP surface binds proteins or causes oxidative effects; (4) the NPs surface produces catalytic effects; (5) NPs change the chemical environment (by influencing pH) (Li et al., 2015). Most studies have concluded that a major cause of phytotoxicity of metal based nanoparticle is the release of toxic ions (Yang and Watts, 2005; Lubick, 2008).

For example, some studies concluded that the toxicity of ZnO NPs resulted from the released Zn^{2+} (Lopez –Moreno et al., 2010a, b; Miller et al., 2010). However, other studies concluded that the toxicity of ZnO NPs itself cannot be overlooked (Lee et al., 2010). Lin and Xing (2008) reported that the presence of ZnO NPs negatively affected the morphology of plant roots (Figure 2.1).

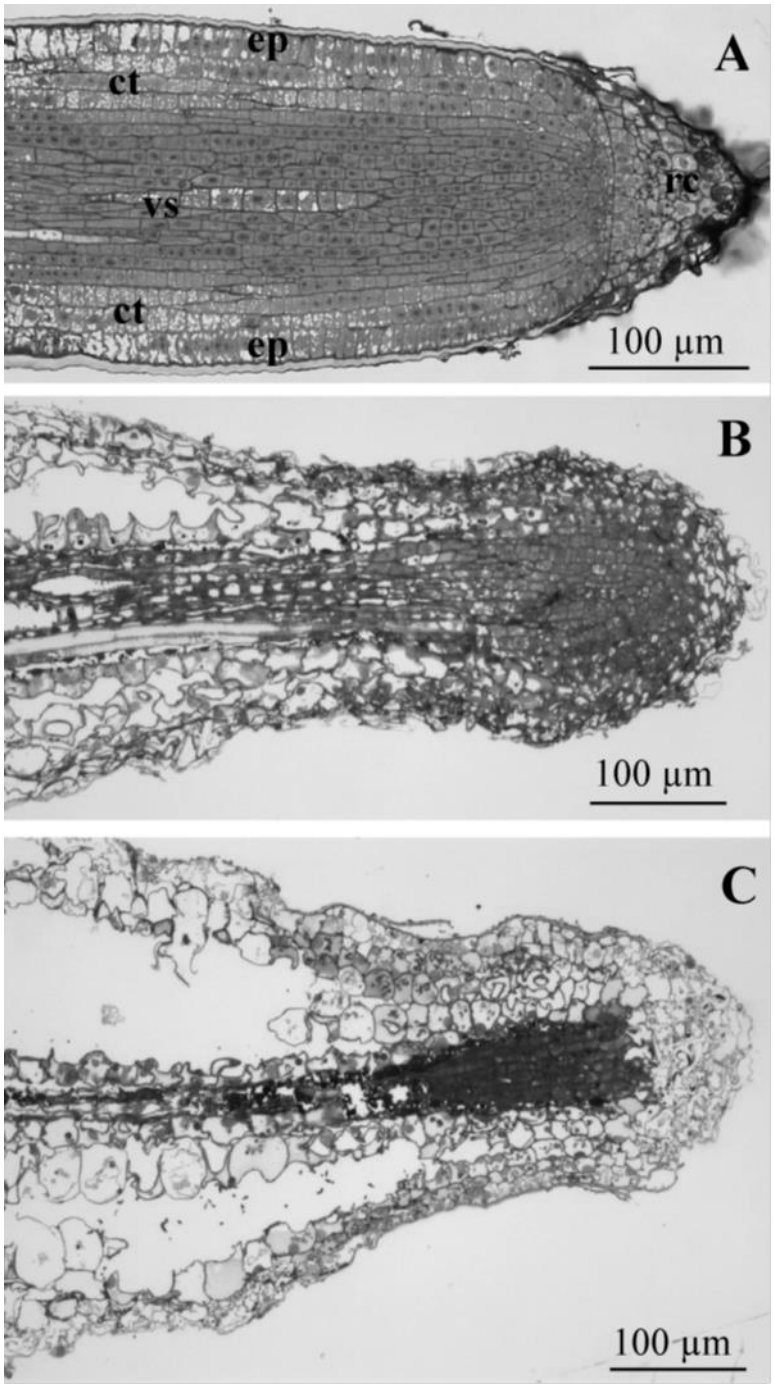


Figure 2.1: Longitudinal sections of ryegrass primary root tips observed under light microscopy, showing the deformity of the morphology of ryegrass root by Zn

species. (a) control, (b) 1000 mg L^{-1} ZnO and (c) 1000 mg L^{-1} Zn^{2+} in hydroponic culture solution. rc: rootcap; ep: epidermis; ct:cortex; vs: vascular cylinder (Lin and Xing, 2008).

2.7.6 Uptake and translocation of nanoparticles in plants

The interactions between NPs and plants are of significance as plants directly interact with the soil, water and atmosphere and transfer contaminants (heavy metals), including nanoparticles between trophic levels in ecosystems (Gardea-Torresdey et al., 2014). Uptake occurs when a material penetrates the cell wall and cytoplasm of the plant (Miralles et al., 2012). Uptake of nanoparticles can occur in the zone of lateral root formation where NPs through the apoplastic bypass enter the xylem (Dietz and Herth, 2011). The plant cell wall serves as a semi permeable barrier that regulates the movement of materials via pores (Lin et al., 2008; Kurepa et al., 2010). The pore sizes of a typical plant cell wall vary with plant species and range from 3 to 8 nm for root hairs. Only NPs or aggregates with diameter less than the cell wall pores can penetrate the cell wall and reach the plasma membrane (Dietz and Herth, 2011). For instance, Au NPs with diameter 3.5 nm were taken up into tomato seedlings whilst those of diamters 18 nm remained agglomerated on the root outer surface (Sabo-Attwood et al., 2012). Nevertheless, some authors have reported absorption and translocation of larger sizes of NPs (Navarro et al., 2008; Corredor et al., 2009; Ma et al., 2010). The nature of the growth matrix and type of plant species appears to affect the uptake of nanoparticles by plants. Lin and Xing (2008) used transmission electron microscopy (TEM) to examine cell internalization and upward translocation of ZnO NPs in ryegrass (*Lolium perenne* L.) grown in solution culture to show that ZnO NPs passed through the epidermis and cortex of the root although, the translocation of Zn to the shoot was not examined. In contrast, plant uptake of NPs was not detected when soil or sand was used as the growth medium. For example, there was no detection of CeO_2 NPs in the leaves of maize (*Zea mays* L.) (Birbaum et al., 2010), ZnO NPs in either roots (Du et al., 2011) or shoots (Dimkpa et al., 2013) of wheat (*Triticum aestivum* L.), and ZnO NPs in stems and pods of soybean (*Glycine max* (L.) Merr) (Hernandez-Viezcas et al., 2013).

2.7.7 Nanoparticles as a remediation tool

In addition to biological methods such as bioremediation, phytoremediation (amongst others) used in remediating contaminated environment, some nanomaterials have recently been applied to environmental remediation as a result of their increased reactivity due to their high surface area per unit of mass (Rickerby and Morrison, 2007; Patil et al., 2016). Nanomaterials such as metal oxides, zeolites, bimetallic nanoparticles, carbon nanotubes and fibers are used for the transformation and detoxification of pollutants (Patilet al., 2016). Nanoscale zero - valent iron (nZVI) is currently been used in reactive barriers for the removal of organic and inorganic contaminants (Figure 2.2) (Nowack, 2008; Garner and Keller, 2014; Tosco et al., 2014; Patil et al., 2016). The process of degradation is based on the redox reaction where Fe (iron) donates electrons to the toxic contaminants, thereby reducing the formation of toxic by product. ZVI can decrease dissolved concentrations of chromate, arsenate, selenite, perchlorate and nitrate (Nowack, 2008; Ghasemzadeh et al., 2014). Karn et al. (2009) reported that treatment of contaminated groundwater with nZVI is accomplished in only 1-2 years as compared with a pump- and - treat method which requires 18 years of operation.

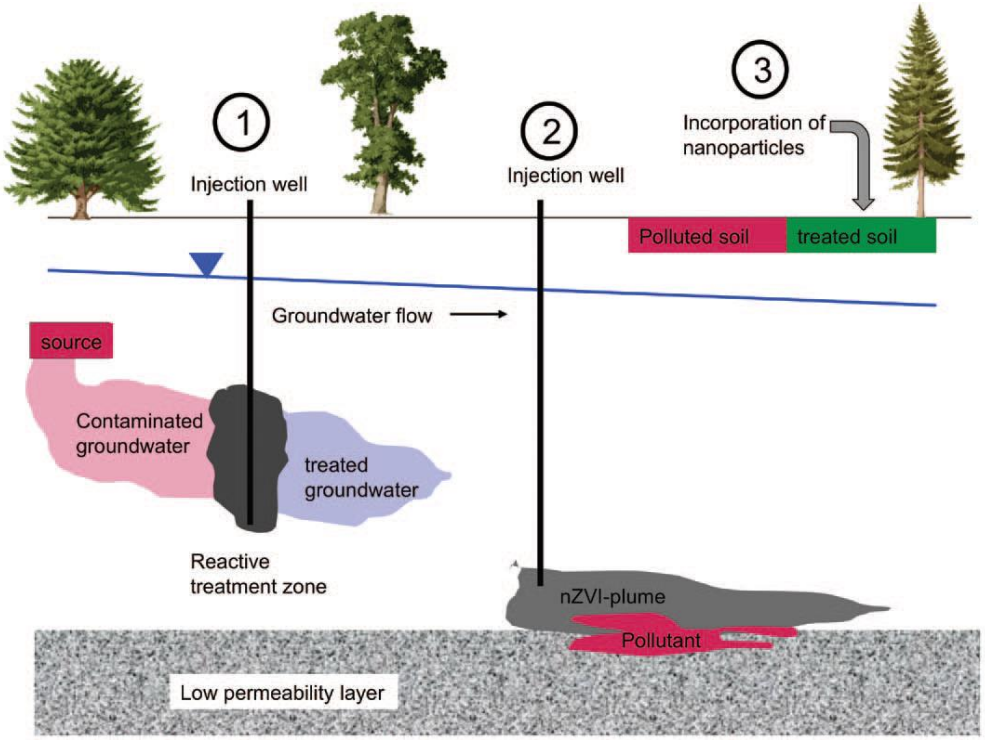


Figure 2.2: In situ technologies for the treatment of contaminated soil and ground water: (1) nZVI is injected to form a reactive barrier; (2) Mobile nZVI is injected to form an nZVI plume; (3) NP is incooperated into topsoil to degrade or adsorb contaminants (Mueller and Nowack, 2010).

2.8 Phytoremediation as a sustainable remediation technology

Phytoremediation is the use of plants that are genetically engineered or naturally occurring plants to clean up contaminated environments. It is considered to be a green revolution for remediating contaminated sites, being a simple, clean and cost effective technology (Liang et al., 2009). It involves the direct use of green plants and their associated microorganisms to remove, reduce, extract or stabilize contamination in surface water, sediments, soils, sludge or groundwater sites (Salt et al., 1998; Sharma and Pandey, 2014). Contaminated sites with low concentration of contaminants and at shallow depths have the most favourable conditions for phytoremediation (Sharma and Pandey, 2014). Phytoremediation can be used to clean up a range of contaminants, including metals, explosives, solvents, and crude oil (Sharma and Pandey, 2014). The success of phytoremediation is mainly dependent on the selective uptake ability of plants in addition to the accumulation, translocation and degradation of contaminants (Tangahu et al., 2011). There are

different mechanisms by which phytoremediation is achieved, including: rhizofiltration, phytostabilization, phytodegradation and phytovolatilization. These phytoremediation processes are described below.

2.8.1 Rhizofiltration

Rhizofiltration is the use of plant roots to concentrate and sorb contaminants from solution (Dushenkov et al., 1995; 1997). Rhizofiltration is a promising technology to remove radionuclides and heavy metals from contaminated water, aqueous waste streams and effluents (Lee and Yang, 2010). The plants used are first acclimated to the contaminant and are not planted directly into the contaminated medium. Plants are hydroponically grown in clean water to form a large root system which further acclimatises when grown in polluted water before planting in the polluted area (Dushenkov et al., 1995). The large roots uptake the contaminant in the polluted water and the roots are harvested. Roots of hydroponically grown higher plants, e.g. sunflower, soybean, bean, grasses and Indian mustard, have been used to absorb heavy metals (cadmium, chromium, nickel, zinc, lead and uranium) and radionuclides (strontium and caesium) from polluted effluents (Dushenkov et al., 1995; 1997; Dushenkov and Kapulnik, 2000; Mei et al., 2002; Easpen et al., 2003).

2.8.2 Phytostabilisation

Phytostabilisation is a plant based remediation measure to reduce the mobility of contaminants in soil (Galende et al., 2014). It involves the use of plant roots or whole plants to prevent the migration of contaminants through soil dispersion, wind, water erosion and leaching. Contaminants in the soil are immobilised through precipitation in the plant root zone, adsorption onto roots, accumulation or absorption by roots (Adreazza et al., 2011). Phytostabilization has been applied in remediation of mine soils (Grandlic et al., 2009) and soils contaminated with heavy metals.

2.8.3 Phytodegradation (Phytotransformation)

Phytodegradation, also known as phytotransformation is the degradation of organic contaminants (e.g. trichloroethylene, polycyclic aromatic hydrocarbons, explosives, herbicides, amongst others) through internal or secreted enzymes of plants (Doty, 2008). Nitroreductase, nitrilase, dehalogenase, peroxidase and phosphatase are some of the enzymes used for phytodegradation. Plants such as the hybrid poplars (Newman et al., 1997), and the tropical leguminous tree *Leucocaena leucocephala* (Doty et al., 2003) have been frequently used for phytodegradation. Phytodegradation is limited to the removal of organic pollutants only because heavy metals are non-biodegradable (Ali et al., 2013).

2.8.4 Phytovolatilization

Phytovolatilization is a remedial measure which involves the use of plants to remove contaminants from soil and water by transforming them into a volatile form (Wang et al., 2012). The contaminants are taken up by plant roots and are then transported through the xylem and released from the cellular tissues into the atmosphere (Wang et al., 2012). However, this measure is only feasible for elements with a volatile form, e.g. Hg.

2.8.5 Rhizodegradation

Rhizodegradation is a form of phytoremediation that involves the degradation of organic contaminants in the soil by soil microbes in the rhizosphere of plants. Plants root exudates (e.g. amino acids, carbohydrates) provide a source of nutrients and energy to the entire rhizosphere microbial population which stimulates increased activity aiding in the biodegradation of some organic contaminants (Susarla et al., 2002). However, this measure is only feasible for organic contaminants and not heavy metals.

2.8.6 Phytoextraction

Phytoextraction, also known as phytoaccumulation is a promising technology for the remediation of contaminated soil (Zhang et al., 2010). It involves the use of hyperaccumulator species (plants that take up and accumulate large quantities of

toxic metals at levels 100-1000 fold than those measured in non-accumulator plants) (Yang et al., 2005) to remove metals from contaminated soil and transfer them to their shoots, which is followed by the seasonal harvesting of plant biomass, until the soil heavy metal concentration decreases (Wei et al., 2008). The two basic types of phytoextraction and the mechanisms involved are shown in Figure 2.3.

(i) ***Chelate assisted or induced phytoextraction***

This involves the addition of artificial chelates that increase the solubility and mobility of contaminants. Chelators such as ethylenediaminetetra acetic acid (EDTA) applied to Pb contaminated soils, resulted in an increased amount of bioavailable lead and greater Pb accumulation in plants (Huang et al., 1997). Marques et al. (2009) reported that Zn concentrations in water extracts of soils, collected during plant harvest were significantly increased 4.0 and 3.1 - fold following the addition of ethylenediamine disuccinic acid (EDDS) or EDTA respectively. The addition of EDTA and EDDS in metal contaminated soil reduced arbuscular mycorrhizal fungi (AMF) colonisation of Zea mays and *Nicotiana tabacum* (Chen et al., 2003; 2004; Wu et al., 2004). For induced phytoextraction to be effective, high plant biomass is required before chelate application. This may be difficult if the soil is heavily contaminated with Zn, Cd, or Cu which are more bioavailable and phytotoxic than Pb (Lombi et al., 2000).

(ii) Continuous phytoextraction

This involves the natural ability of plants to extract and remove contaminants (Salt et al., 1997). Hyperaccumulator plants accumulate metals in their shoots and roots and have an exceptionally high tolerance to heavy metals (Baker et al., 2000). Some hyperaccumulators tend to produce low biomass and are also slow- growing. Brown et al. (1994) estimated that 28 years of *Thlaspi caerulescens* cultivation would be required to extract Zn from a soil containing 2100 mg Zn kg^{-1}. Another limitation is that some metals are immobile in soil and their extraction rate is limited by solubility and diffusion to the root surface (Lombi et al., 2000).

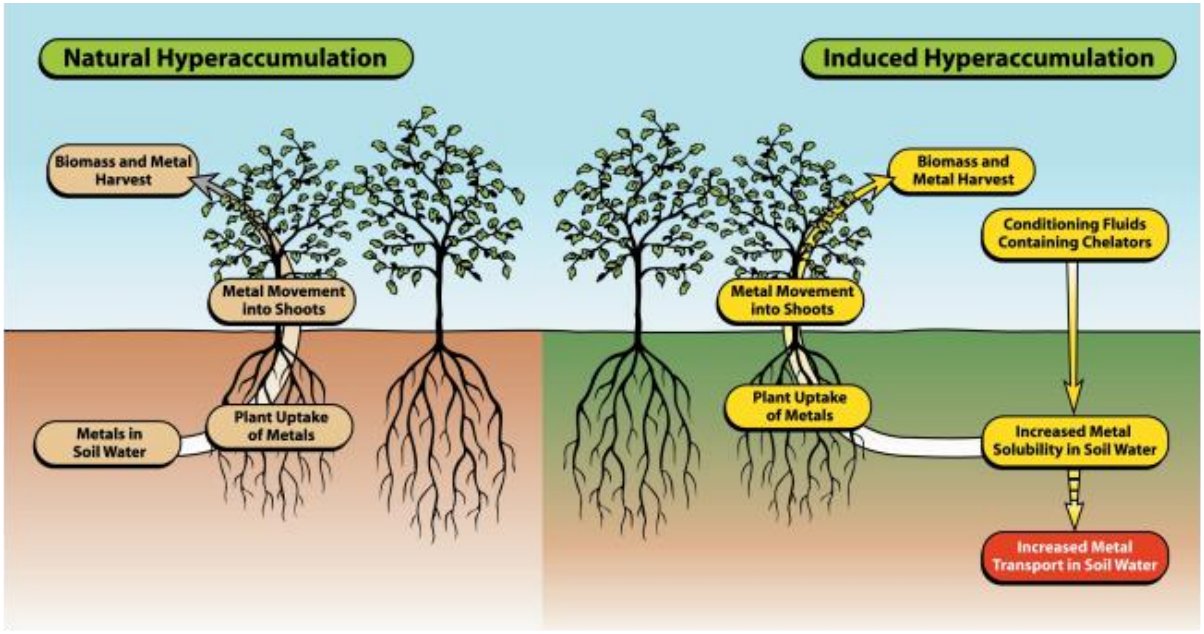


Figure 2.3: Natural and Induced Phytoextraction.
(http://www.bnl.gov/erd/Peconic/Factsheet/Phytoextract.pdf)

2.9 Hyperaccumulator species

Recently, 450 angiosperm species from at least 45 plant families have been shown to be metal hyperaccumulators (Rascio and Navari-Izzo, 2011) in particular *Brassicaceae* and *Asteraceae* (Seth, 2012). The *Brassicaceae* is a large angiosperm (flowering plant) dicot family which belongs to the order Brassicales. It contains 338-360 genera and 3709 species distributed worldwide on all continents except Antarctica (Al-Shehbaz, 1973; Appel and Al-Shehbaz, 2003; Al-Shehbaz et al., 2006). The Brassicaceae have potential to remediate varied environmental contaminants (Milner and Kochia, 2008; Kramer, 2010) and have shown to tolerate trace metals and to accumulate elevated concentrations of metals in the roots and shoots (Kumar et al., 1995; Ebbs and Kochian, 1997). *Brassica juncea* (L.) CZern and Coss are noted for their capacity to tolerate, stabilise, extract and accumulate metals such as Zn, Pb, Ni, Cu, Cr, or Cd (Ebbs et al., 1997; Ebbs and Kochian, 1998; Prasad and Freitas, 2003; Bluskov et al., 2005; Meyer et al., 2008; Purakayastha et al., 2008; Singh and Fulekar, 2012; Adediran et al., 2015).

A number of other plant species as seen in Table 2.2 have also been identified as hyperaccumulators and have been used by researchers to investigate remediation potentials and the processes involved. Often, due to the limitations of phytoremediation (discussed below) these plants are combined with other traditional technologies for cleaning up contaminated environments (Ma et al., 2001; Rosen, 2002; Sasaki et al., 2006; Meda et al., 2007; Shoa et al., 2009). Examples of hyperaccumulators plant species for heavy metal phytoremediation are given in Table 2.2.

Table 2.2: Examples of some metal hyperaccumulator and their bioaccumulation potential (Sarma, 2011; Mahar et al., 2016)

Plant species	**Metals**	**Metal accumulation (mg / kg)**	**References**
Sesbania drummondi	Cd	1687	Israr et al. (2006)
Thlaspi caerulescens	Cd	80	Banasova and Horak (2008)
Corrigiola telephiifolia	Cd	236.2	Kucharski et al. (2005)
Arabis paniculata	Cd	1127	Zeng et al. (2009)
Sedum alfredii	Cd	2183	Jin and Liu (2009)
Solanum photeinocarpum	Cd	158	Zhang et al. (2011)
Pteris vittata	Cr	20, 675	Kalve et al. (2011)
Zea mays L ganga 5	Cr	2538	Sharma et al. (2003)
Phragmites australis	Cr	4825	Calheiros et al. (2008)
Eichornia crassipes	Cr (Vi)	6000	Lytle et al. (1998)
Ipomea alpine	Cu	12,300	Baker and Walker (1989)
Sorghum sudanense	Cu	5330	Wei et al. (2008)

Silene vulgaris	Hg	4.25	Araceli et al. (2002).
Alyxia rubricaulis	Mn	11,500	Chaney et al. (2010)
Seberia acuminate	Ni	250,000	Jaffre et al. (1976)
Berkheya coddii	NI	5,500	Robinson et al. (1997)
Brassica Juncea	Ni	3916	Sarawat and Rai (2009)
Brassica juncea	Pb	10,300	Koptsik (2014)
A.racemosus	Se	14,900	Beath et al. (1937)
Iberis intermedia	Ti	3,070	Leblanc et al. (1999)
Thlaspi calaminare	Zn	21,150	Baker et al. (1994)
Deshampsia cespitosa	Zn	3614	Sakakibara et al. (2008)
Thlaspi caerulescens	Zn	19410	Banasova and Horak (2008)
Sedum alfredii	Zn	13,799	Jin and Liu (2009)
Potentilla griffithii	Zn	19,600	Hu et al. (2009)

2.9.1 Mechanisms of heavy metal uptake and transport

Contaminant uptake is an important process in phytoextraction. The extent to which plants take up metals is dependent on metal bioavailability and concentration in soil which is influenced by redox potential, pH, presence of organic matter, concentrations of other elements and temperature (Benavides et al., 2005). Metal uptake by roots may take place at the apical region or from the root surface (Setia et al., 2008). For effective metal uptake, metals that are fixed in mineral form are first mobilised into soil solution. Plants tend to achieve this by secreting phytosiderophores into the rhizosphere to solubilise and chelate metals that are bound (Wu et al., 2004). The mobilized metal which is bound to the cell wall is captured by the root cells (Ghosh and Singh, 2005). Inside the plant, the apoplast is the free diffusional space outside the plasma membrane which aids in the transport of solutes and water across a tissue while the symplastic pathways aid in the

movement of water from cell to cell in the cytoplasm through the plasma membrane (Raskin et al., 1997; Ghosh and Singh, 2005; Thakur et al., 2016). Apoplastic and symplastic pathways are two alternative pathways for metal uptake in plants (Seith et al., 2008). Metal ions require membrane transporter proteins for transportation from the root endodermis into the root xylem (Pilon- Smits, 2005). Although some metals are chelated by organic acids (citrate, histidine) during xylem (Pickering et al., 2000), it is still unclear which metal ion transporter proteins are exported to root xylem (Pilon- Smits, 2005). Specific transporters for metal hyperaccumulators have been reported. In *Arabidopsis thaliana*, *AtHMA1-AtHMA4* transporters are involved in root-to-shoot translocation of Zn, Co, Cd, and Pb (Mills et al., 2003; Hussain et al., 2004; Verret et al., 2004; Eren and Arguello, 2004; Morel et al., 2009; Wong and Cobbett, 2009). In *Arabidopsis halleri*, *AhHMA4* contributed to Zn translocation from root to shoot (Talke et al., 2006). There is considerable evidence that arsenic (As) can enter plant root as arsenate via transporters of the chemical analogue - phosphate (Meharg and Hartley-Whitaker, 2002; Rascio and Navari-Izzo, 2011).

2.9.2 Mechanisms of metal detoxification or tolerance in plants

Microelements such as Cu, Zn, Mn and Ni are vital for plant development and growth but these metals also tend to be toxic at high intracellular concentration. At the cellular level plants have several mechanisms to detoxify metals. These include regulation of ion efflux (stimulation of transporter activity at low intracellular ion supply and inhibition at high concentrations) and extrusion of intracellular ion supply back into the external solution (Lombi et al., 2002; Long et al., 2002). Another detoxification mechanism reported in metal hyperaccumulator plant species that are capable of taking up metals in thousands of ppm is complexation with ligands. It has been shown that a Ni hyperaccumulator plant *Thlaspi goesingense* was highly tolerant, as a result of Ni complexation by histidine (Kramer et al., 1996). Zn has been shown to be mainly complexed with histidine (70%) in plant roots (Salt et al., 1999), whilst in *Noccaea caerulescens* it occurred more as Zn-O complexes (Zn complexed with carboxylic or aqueous ligands) (Kupper et al.,

2000). The synthesis of phytochelatins and antioxidants are also plant tolerance mechanisms against metal induced oxidative stress (Seth et al., 2007, 2008; Seth, 2012).

2.9.3 Factors affecting metal uptake by plants.

Soil factors

Soil characteristics such as organic matter content, cation exchange capacity, soil pH, and sorptive capacity (described above) have a strong effect on plant uptake of metals. Not all forms of metal are available for uptake. The forms of metals that are readily available are those which are dissolved in the soil pore water (Sauve et al., 2000), whilst metals that are a core part of solid phase minerals are not phytoavailable (Lombi et al., 2003).

Differences between plant species

Concentrations of metals in plants growing in the same soil vary between plant species (Hamon et al., 1997). Consequently, plant selections are vital and those plant species that can hyperacccumulate heavy metals and produce large amount of biomass are suitable for phytoextraction.

Root zone

The root zone is an important aspect that might have an effect on uptake efficiency. (Tangahu et al., 2011). Plant root can influence heavy metal phytoavailability (Elekes, 2014) by modifying the soil properties in the rhizosphere (Brown et al., 2003). Root lengths differ between plant species (Hamon et al., 1999; Negri et al., 2003). The roots of non –metal-tolerant species have been shown to avoid areas of soil highly contaminated by toxic metals thus reducing the heavy metal uptake in soil (Schwartz et al., 1999).

Transpiration

Transpiration rates differ and are dependent on humidity, soil moisture content and plant species (Larcher, 1995). Plant transpiration rates will influence metal uptake in that the more plants transpire, the greater the mass flow of soil solution to the root surface. This increases the concentration of the metal at the root surface thus enhancing metal uptake (Hamon and McLaughlin, 2003).

2.9.4 Advantages and limitations of phytoextraction

Phytoextraction is an environmentally aesthetically pleasing (since it uses plants) and cost effective technology which is designed to concentrate metals in plant tissue (Salt et al., 1995). There is less environmental disruption as the treatment preserves the top soil that can be recovered for agricultural use (Tangahu et al., 2011). The high yield of biomass is effective in reducing high concentrations of heavy metals to a low level. Metal-rich plant residue can be reused (Tangahu et al., 2011), e.g. Bio-ore (Brooks et al., 1998; Nick and Chambers, 1994), and smelting (Koppolu et al., 2003).

Although, the effectiveness of phytoextraction is limited by a number of factors including: the low availability of heavy metals in soil, low biomass and slow growth of most hyperaccumulators (Sheng et al., 2012; Mahar et al., 2016). Stressors such as weeds, plant pathogens, and variation in nutrients, precipitation and temperature may affect plant growth (Gerhardt et al., 2009). The consumption of contaminated plants by wildlife may be possible (Ghosh and Singh, 2005).

2.10 Plant Growth Promoting Bacteria (PGPB)

Use of plant growth promoting bacteria (PGPB) may overcome some of the limitations of phytoextraction (Burd et al., 2000). Plants are subjected to abiotic and biotic factors that influence their production rate, root development and growth (Haghighi et al., 2011). Microorganisms such as protozoa, algae, fungi, bacteria, and actinomycetes are numerous in the soil (Parsaeimeh, 2009; Homayoun, 2011; Ordookhani, 2011). Bacteria are the most abundant, with 1 g of soil containing 10^8 - 10^9 bacterial cells (Glicks, 2012). The number and type of bacterial found in soil is influenced by soil conditions including moisture, temperature and plant species

amongst others. Bacteria are not evenly distributed in soil, with greater concentration found around the roots of plants (rhisozphere) than in bulk soil (Glicks, 2012). Bacteria that provide benefits to plants either by free-living in the soil or by symbiotic relationships with plants are known as plant growth promoting bacteria (PGPB) or plant growth promoting rhizobacteria (PGPR). These plant associated bacteria migrate from the bulk soil to the rhizosphere where they colonise the roots of the plant and rhizosphere (Kloepper and Schroth, 1978; Kloepper et al., 1991). Diverse symbiotic (*Rhizobium, Bradyrhizobium, Mesorhizobium*) and non-symbiotic (*Pseudomonas, Bacillus, Klebsiella, Azotobacter, Azospirillium, Azomonas*) rhizobacteria are being used as bio-inoculants to promote plant growth and development under various stresses, such as heavy metals (Wani and Khan 2010; Ma et al., 2011; Adediran et al., 2015), herbicides (Ahemad and Khan, 2011), salinity (Mayak et al., 2004).

Rhizobium – legume symbiosis has been examined extensively. The N_2 fixed by rhizobia in legumes can also benefit non – legume plants through direct transfer of biologically fixed N (Snapp et al., 1998; Hayat et al., 2008a, b). Rhizobia have been isolated as natural endophytes from roots of non-legume species such as cotton, sweet corn (Mclnory and Kloepper, 1995), maize (Martinez-Romeo et al., 2000) and canola (Lupwayi et al., 2000). Wiehe and Holfich (1995) reported that strain R39 of *Rhizobium leguminosarum* bv. *trifolii* increased under field conditions in the rhizosphere of host legume (lupin and pea) and non- legumes including corn (*Zea mays*, rape (*Brassica napus L*) and wheat (*Triticum aestivum*). The ability of rhizobia to colonize roots and localise internally in tissues including the xylem has been demonstrated in a number of studies (Spencer et al., 1994). The plant growth promoting effect of *Rhizobium leguminosarum bv. trifolii* on non- legume plant has been reported to be similar to *pseudomonas* species (Holich, 2000; Adediran et al., 2015).

2.10.1 Interaction in the rhizosphere

The rhizosphere, is the zone of soil surrounding living plant roots subjected to the direct influence of root activity (Hinsinger et al., 2005). It has been redefined to include the volume of soil where physical, chemical and biological parameters are

strongly influenced by surrounding plant roots (Badri et al., 2009). The rhizosphere can be as thin as a few µm or as thick as a few cm (Alford et al., 2010). Soil, microorganisms and plants are in a mutual relationship. Plants are a major source of organic nutrients in soil and as driving force for microbial activity, whilst soil microbes interact with the plant root and can modulate plant responses to biotic and abiotic stress (Minz et al., 2013). Biogeochemical processes have been shown to differ between the bulk and rhizosphere soil (Hinsinger et al., 2005; Fageria and Stone, 2006; Watt et al., 2006). The rhizosphere effect differs with plant species (due to differences in the nature of exudates, root system complexity), soil type and properties (Hinsinger and Courchesne, 2008). In the rhizosphere, biochemical interaction takes place, influencing plant growth and yields (Haghighi et al., 2011). Plants exude diverse compounds, such as organic acids, amino acids, proteins (enzymes), phenolic compounds, and polysaccharides, which are dependent on photosynthesis rate, soil type, plant physiological condition, age and substrate availability (Kidd et al., 2009). It has been estimated that about 10 to 40% of assimilated carbon can be released directly into the soil through root exudates (Singer et al., 2003; Bias et al., 2006). Root exudates may also influence the behaviour of trace metals and nutrients either by reducing or enhancing their availability: indirectly through their effects on microbial activity, root growth patterns and physical and chemical properties of the rhizosphere; or directly by affecting acidification, chelation, precipitation and redox reactions (Uren and Reisenauer, 1998; Tao et al., 2004; Jing et al., 2007).

2.10.2 Importance of PGPB in metal contaminated soil

Different types of PGPB, including *Flavobacterium, Agrobacterium, Azotobacter, Acetobacter, Arthrobacter, Bacillus, Burkholderia, Chromobacterium, Pseudomonas, Azospirillum, Xanthomonas, Klebsiella, Erwinia, Micrococcus* (Misko and Germida, 2002; Gary and Smith, 2004), have been reported to have advantageous effects on plants in metal contaminated environments (Tokala et al., 2002; Dimpka et al., 2008a, b; 2009a, b). Most PGPB can degrade contaminants or prevent metal accumulation in specific plant tissue (see Table 2.3 for examples). For example,

Arthrobacter mysorens was found to be resistant to lead and cadmium, preventing accumulation of these heavy metals in plant tissue also enhancing the growth of barley in heavy metal contaminated soil (De-Bashan et al., 2011). Khan et al. (2009) reported that *Bacillus* strains that were isolated from chromium contaminated soil reduced highly toxic Cr^{6+} to less toxic Cr^{3+}.

Isolation of rhizosphere bacteria and supporting them with appropriate metabolites that allows the synthesis of natural chelators can improve metal bioavailability and reduce phytotoxicity (De-Bashan et al., 2011). Metal-resistant endophytic bacteria reduced metal toxicity in plants (Weyens et al., 2009). Li et al. (2007) reported that uptake of zinc and cadmium by *Sedum alfredii* can be increased following the inoculation with endophytic bacteria.

Several mechanisms have been suggested by which PGPB can promote plant growth and health. These includes: increasing availability of nutrients such as through biological nitrogen fixation, phosphate solubilisation and mineralization; production of growth regulators, such as indole, cytokines, or gibberellins, biological control; and enhancement of water uptake, (Ma et al., 2011; Rojas-Tapias et al., 2012).

Table 2.3: Examples of studies demonstrating the role of PGPB in metal contaminated soil (Zhuang et al., 2007)

Bacteria	Plant	Heavy metal	Condition	Role of PGPB	Reference
Azotobacter chroococcum HKN-5. *Bacillus megaterium HKP-1*	*Brassica Juncea*	Lead, zinc	Pot experiments in greenhouse	-Stimulated plant growth. -Protected plant from metal toxicity.	Wu et al. (2006)
Bacillus subtillis SJ-101	*Brassica juncea*	Nickel	Pot experiments in growth chamber	Facilitated Ni accumulation	Zaidi et al. (2006)
Brevundimonas sp. KR013. *Rhizobium leguminsaum bv. trifolii* NZP561 *Pseudomonas sp*. KR017	None	Cadmium	Culture media	-sequestered Cd directly from solution	Robinson et al. (2001)
Kluyvera ascorbata SUD165 Kluyvera ascorbata SUD 165/26	Indian mustard Canola Tomatoes	Nickel, lead, zinc	Pot experiment in growth chamber	-Both strains decreased some plant growth inhibition by heavy metals. -No increase of metal uptake with either strain over non inoculated plants	Burd et al. (2000)

2.10.3 Plant growth promoting factors

Plant-associated bacteria synthesise phytohormones, chemical messengers that affect a plant's ability to respond to its environment. Phytohormones, such as auxins, gibberellins, indole-3-acetic acid (IAA) and, cytokinins influence reproduction, growth, germination, and protect plants from biotic and abiotic stress (Taghavi et al., 2009). PGPB also control stress through the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase which promotes growth and development by decreasing plant ethylene levels (Glick et al., 2007). Studies have shown that plants inoculated with PGPB containing ACC grew well in metal polluted soils (Rodriguez et al., 2000; Ma et al., 2011; Zhang et al., 2011a, b). Pseudomonas bacteria such as *P. fluorescens* and *P. putida* produces auxins and increase plant yields (Abbas-Zadeh et al., 2010; Glick et al., 1999). Rhizobia bacteria release IAA that promotes growth and pathogenesis in plants (Mandal et al., 2007b).

2.10.4 PGPB as biological control agents

PGPB, such as *Pseudomonas sp*., are potential biocontrol agents protecting plants from insects and pests as well as viral and fungal diseases. This is mediated by the production of bacterial allelochemicals including detoxification enzymes, lytic enzymes, iron-chelating siderophores, antibiotics and biocidal volatiles by the PGPB (Compant et al., 2005).

2.10.5 Production of siderophores by PGPB

Iron is an essential growth element and therefore limiting when not bioavailable. In soil, under aerobic conditions, most iron exists in an insoluble form (Fe^{3+}) which is not easily available for plants and microbes (Gadd, 2010; Rout and Sahoo, 2015). To meet this iron requirement, PGPB secrete or produce low molecular weight compounds known as siderophores which have a high affinity for Fe^{3+} ions. Siderophores aid in the sequestering and transport of iron to the cell (Rodriquez and Smith, 2003). PGPB that reside in the rhizosphere soil are well recognised for their role in heavy metal phytoextraction (Dimkpa et al., 2009a; 2009b; Rajkumar et

al., 2010). Dimkpa et al. (2009b) reported that siderophores produced by *Streptomyces tendae* F4 enhanced uptake of Cd by sunflower plants. Braud et al. (2009) also reported that the production of Pyoverdin and Pyochelin (siderophores) by *Pseudomonas aeruginosa* increased the concentrations of Cd and Pb in soil making them available for maize uptake. In contrast, other studies have demonstrated that the production of siderophores by PGPB reduces the uptake of metals by plants. For instance, Sinha and Mukherjee (2008) found that inoculation with *P. aeruginosa strain KUCd 1* reduced Cd uptake in shoots and roots of *Brassica juncea* and *Cucurbita pepo.* Similarly, Tank and Saraf (2009) reported reduced Ni-uptake in chickpea plants but increased plant growth, following inoculation with Ni-resistant- siderophore- producing *Pseudomonas*. This conflicting view may be due to differences in plant type, metal bioavailability, and plant ability to transport metals from roots to shoots (Rajkumar et al., 2012).

2.11 Conclusion

Whilst Zn is an essential micronutrient, its phytotoxicity and impact on the entire environment is a global concern. Zn in contaminated soil is non-biodegradable and thus persists for a long time, but changes in its chemical form and bioavailability are possible. Phytoremediation offers a possible low cost method and seems to be beneficial in restoring Zn contaminated environments (Adediran et al., 2015). Plants are an essential base component of all ecosystems and play a critical role in the fate and transport of metal contaminants including NPs through plant uptake and bioaccumulation (Monica and Cremonini, 2009). Although information exists in the interaction between metals and plants. Limited knowledge about the uptake of nanoparticles (NPs), the mechanisms by which they penetrate plants, their interactions with soil and toxicity is still hindering their full application (Ma et al., 2010; Marmiroli et al., 2014). The majority of studies investigating the effects of NPs on plants have been conducted in vitro (hydroponics or culture media) and very few studies have investigated NPs bioavailability to plants in soil. Thus, there is a need to study the uptake, translocation and biotransformation of NPs in the soil environment so as to inform associated risk.

The addition of plant growth promoting bacteria increases Zn bioavailability in soils which improves phytoextraction of Zn contaminated soils. The interaction between PGPB and toxic metals in soil may alter their physical and chemical state of metals which influences plant growth, activity and survival. Despite demonstration of the potential of managing plant-microbe-soil interactions to enhance remediation of contaminated soils, much still remains unknown. Understanding these interactions is needed to assess its potential effect on plant dynamics and thus on the effectiveness of phytoremediation.

The next chapter of this thesis explain the experiments specifically designed to examine the effects of Zn speciation on roots and shoots of *Brassica juncea* (L.) Czern in Zn contaminated soil that will provide insight into the physiology of Zn hyperaccumulation in this plant. This will provide avenues for the improvement of phytoremediation technology.

Chapter 3

3 Materials and methods

Data for this thesis were obtained from greenhouse experiments involving plant and soil samples. Subsequent laboratory analyses were performed on soil and plant samples from these experiments. This chapter focuses on the choice of materials, sample preparation, data processing and test procedures with justification of methods for each experiment. Specific information on materials and methods for each experiment is also highlighted in each results and discussion chapter.

3.1 Research materials

3.1.1 Selection of metal and metal based nanoparticles

This study focused on contamination of soil by soluble Zn (in the form of $ZnSO_4$. $7H_2O$), and particulate ZnO and ZnS. ZnO nanoparticles have been used in a variety of applications including, UV absorbing materials, gas sensors and coating for solar cells (Hernandez-Viezcas et al., 2011) and due to concerns about the environmental impact of nanoparticles, ZnO NPs have been included for testing by the Organisation for Economic Cooperation and Development (OECD) (Kahru and Dubourguier, 2010). ZnS has been used as a model nanoparticle in diverse applications including light emitting diode (LED), lasers, infrared windows and solar cells (Singh et al., 2016; Unmartyotin et al., 2016).

3.1.2 Selection of desired plants

Criteria for selecting a plant species for experiments were a plant with known metal remediation characteristics and which occurs naturally in the environment and is readily available. The plant chosen for this study was *Brassica Juncea* (L.) *Czern, also* known as Indian mustard and a member of the Brassicaceae family. It is an annual plant with a short growing season and has been identified as a hyperaccumulator because of its fast growth rate, high metal accumulation and translocation of Zn from root to shoot (Wang et al., 2009; Qu et al., 2012; Adediran et al., 2015). Seeds

of *Brassica juncea* were purchased from Sow Seeds Ltd., UK, stored in a clean plastic bag and kept in the dark at room temperature (14 -16 ^{o} C) until required for use.

3.2 Selection of plant growth promoting bacteria

Two plant growth-promoting bacteria (PGPB) were selected for this study. Ensuring plant growth promotion and root colonisation are the main criteria for selection of PGPB for this study. The selected PGPBs are discussed below.

• ***Rhizobium leguminosarum bv. trifolii***

Rhizobium leguminosarum bv. trifolii bacteria are of the genus *Rhizobium*. These soil based bacteria are small, rod shaped bacteria that have the ability to produce nodules on the roots of leguminous plants and perform nitrogen fixation. Khan et al. (2009) reported that different nitrogen fixers, such *as Rhizobium* sp. RP5, *Rhizobium* sp. RL9, and, *Bradyrhizobium* sp. RM8, were tolerant to Ni- Zn mixtures, and Zn. Wani et al. (2008) also reported that inoculation of *Rhizobium* sp RP5 protected pea plants against the toxic effects of Zn and Ni. A recent study reported that, *Rhizobium leguminosarum bv. trifolii* colonised plant roots, promoted plant growth and enhanced Zn accumulation in a non-leguminous plant *(Brassica juncea*) (Adediran et al., 2015; 2016).

Rhizobium leguminosarum bv. trifolii (strain WSM1325) isolated from the rhizosphere of a clover plant (School of Biological Sciences, University of Edinburgh, UK) was used in this study.

• ***Pseudomonas brassicacearum***

Pseudomonas brassicacearum are a group of PGPB which are major root colonisers of *Brassica* plant species (Achouak et al., 2000; 2004), promoting root elongation, and increasing root and shoot biomass (Belimov et al., 2001; Safronova et al., 2006; Adediran et al., 2015) and which have been shown to be resistant to Zn (Duan et al., 2013). It has been reported that *Pseudomonas brassicacearum* was isolated from the rhizoplane of a *Brassica* plant (Ortet et al., 2011; Loewen et al., 2014).

P. brassicacearum subsp. *brassicacearum* (strain DBK11), obtained as a lyophilisate from the German collection of microorganisms and cell cultures (Leibniz Institute, DSMZ Germany) with DSM number 13227, was used in this research.

3.3 Sources and preparation of experimental materials

3.3.1 Zinc forms

Zinc sulphate ($ZnSO_4.7H_2O$) and ZnO nanoparticles were purchased from Sigma Aldrich UK (< 100 ml of 50% wt. solution in water, ± 35 nm average nanoparticle size) and stored according to the vendor's instructions. ZnS nanoparticles were synthesised in the laboratory using a chemical precipitation method (Ganguly et al., 2014) as described below.

3.3.2 Preparation and characterization of ZnS nanoparticles

The chemicals used in the synthesis of ZnS nanoparticles were of analytical grade and used without further purification.

ZnS nanoparticles were made from 1 M aqueous solution of Na_2S and 1 M aqueous solution of $ZnCl_2$ and the addition of 0.01 M Tris-buffer. Na_2S solution was added dropwise to $ZnCl_2$ solution, whilst stirring the mixture continuously with a magnetic stirrer until a milky white precipitate of ZnS was formed (Equation 3.1).

$$ZnCl_2 + Na_2S \rightarrow ZnS + 2\,NaCl \qquad \text{(Equation 3.1).}$$

The precipitate was allowed to settle, centrifuged at 3000 g (Sharma, 2012), washed four times with deionised water and freeze dried. The fine powder samples obtained were characterised by several methods as described below.

(i) The mineralogy and crystal structure of the ZnS nanoparticles was examined using X-ray diffraction (XRD). 0.1 g of dry powdered ZnS sample was measured on a Bruker D2 PHASER diffractometer fitted with a LynxEye detector and operating in a flat plate mode using Ni -filtered Cu K-alpha radiation (λ= 1.54060 Å) (start: 5 degrees; end: 90 degrees; time per step: 0.3 seconds).

(ii) The crystallite size was calculated from the Debye-Scherrer formula (Hammond, 2009),

$$D = \frac{K\lambda}{\beta cos\theta} \qquad \text{(Equation 3.2)}$$

where D is the mean diameter of the crystallite (nm), k is a constant related to the dimensionless shape (0.94), λ is the X-ray wavelength (Å), β is the full width at half the maximum intensity (radians, r) and θ is the corresponding diffraction angle (^{o}).

(iii) Microstructural investigation was carried by transmission electron microscopy (TEM). A 2 µL drop of ZnS nanoparticles suspension were allowed to dry slowly on a carbon -coated copper grid and viewed in a Philips CM120 Transmission electron microscope operated at 200 kV. Images were taken on a Gatan Orius CCD camera.

3.4 Soil selection and characterisation for growth experiments

Soil amended with peat has been reported to influence metal speciation by modifying metal mobility and availability due to a high organic matter content (Al-Chami et al., 2013). Organic matter can also influence sulfur speciation and, since ZnS was one of the Zn forms used in the study, soils containing peat were avoided. Instead, topsoil (made up of clay, silt and sand) without peat amendment was used as a natural soil that contains all nutrients suitable for plant growth. Topsoil encourages rooting, airflow and drainage and is also a representative of field conditions.

Westland topsoil was purchased from Dobbies Garden Centre, Edinburgh, UK. The soil was amended with 10 % by volume sand to improve drainage.

3.4.1 Preparation of soil samples for characterisation

Soil samples were prepared for determination of physical and chemical properties by first air drying at approximately 30^{o}C for a minimum of 48 hours. The air dried soil was then crushed, and passed through a 2 mm stainless steel sieve to disaggregate clumps and remove coarse debris present.

3.4.2 Determination of soil pH

Soil pH was determined by preparing a 1:2 soil: water suspension by weight in which 10 g of sieved air dried soil was weighed into a 50 ml beaker and 20 ml of distilled water was added. The mixture was stirred and shaken for 10 minutes, allowed to stand for another 15 minutes to enable particles to settle. The pH meter was calibrated using buffer solutions at pH 7.0 and 4.0 before the electrode was immersed in the slurry to record a stable pH reading. Soil pH measurement were conducted on three replicate samples.

3.4.3 Determination of soil moisture content

20 g of fresh soil was weighed in triplicate into a weighed evaporating basin. The sample was placed in an air-circulating oven at 105 ^{o}C overnight. The basin was removed and cooled in a dessicator and reweighed. Percentage soil moisture content was calculated from loss in weight as shown below.

% soil moisture =

$$\frac{loss\ in\ weight\ on\ drying(g) \times 100}{initial\ sample\ weight\ (g)} \qquad \text{(Equation 3.3)}$$

3.4.4 Determination of soil organic matter content by loss of ignition

Ignition of soil at high temperature will result in a loss of weight due to loss of organic matter. 5 g of oven dried soil was weighed in triplicate into a weighed dry crucible and placed in a muffle furnace at 450^{o}C for four hours. The sample was transferred to a desiccator to cool to room temperature and weighed. The % organic matter by loss on ignition (LOI) was calculated by:

% organic matter (LOI) =

$$\frac{oven-dried\ weight\ (g)-furnace\ weight\ (g)}{oven-dried\ weight\ (g)}\times 100 \qquad \text{(Equation 3.4)}$$

3.4.5 Determination of soil total organic nitrogen content

Total organic nitrogen in the soil was determined by using a wet method as proposed by Kjeldahl (1883). 1 g of finely ground soil was weighed and transferred to a 250 ml glass Pyrex flask and placed in a Buchi digest system (K-437). Three replicate samples and two blanks (without soil) were prepared. 20 ml of concentrated sulphuric acid was added and the suspension heated for at 370^{o}C at 3 h. The sample was cooled and filtered with Whatman No. 44 filter paper and diluted to the 50 ml with deionised water. Nitrogen concentration was determined colorimetrically using a Bran- Luebbe auto analyser series 3 and measuring absorption at 660 nm.

3.4.6 Determination of soil available phosphorus and potassium

5 g air-dried soil was weighed into a 150 ml Erlenmeyer flask. Four replicate samples and two blanks (without soil) were prepared. 100 ml of 2.5 % acetic acid was added and the samples were shaken in an orbital shaker for 2 h. The sample was filtered through Whatman No. 54 filter paper, discarding first few ml of the filtrate. Orth-ophosphate in the filtered extract was determined colorimetrically using a Bran – Luebbe auto analyzer series 3 and measuring at 660 nm for phosphorus.

The same and the extract was analysed by atomic absorption spectrophotometry at 766.5 nm (Thermo ICE 3000 series) for soil available potassium.

3.4.7 Determination of total soil zinc, sodium, iron, calcium, magnesium, manganese

These elements are chemically analysed from soil to determine their available content within the soil sample. 0.2 g of dry soil was weighed into crucibles, and placed in a muffle furnace at 500 ^{o}C for 3 hrs. Three replicate samples and two blanks (without soil) were prepared. 5 ml of 37 % HCl (concentrated) was added to the cool sample, covered with a watch glass and heated on a steam bath for 15

mins. 1 ml of 30% HNO_3 (concentrated) was added and allowed to evaporate to dryness. 2 ml of 6M HCl was added and the crucible swirled to dissolve the residue. Then 10 ml of deionised water was added and the crucible warmed gently. Extracts were filtered using Whatman No. 44 filter paper into volumetric flasks and made up to 50 ml and analysed in an atomic absorption spectrophotometer (Thermo ICE 3000 series) to determine concentrations of Zn, Na, Fe, Ca, Mg, Mn using calibration standards.

3.5 Bacterial zinc resistance tests

The degree to which soluble Zn, ZnS and ZnO nanoparticles are toxic to the PGPB used in this study (*Pseudomonas brassicacerum* and *Rhizobium trifolii)* was established by conducting dose-response growth experiments in medium and dose-response viability experiments (using colony forming units, CFU) following incubation in (a) minimal medium and (b) Hoagland solution. The rationale behind this approach was to use minimal medium and reduce bacterial growth rate, which is known to increase resistance. CFU experiments involved exposing the bacteria to Zn concentrations ranging from 0 (controls) to 800 mg L^{-1} in each medium for different periods (up to 3 days), performing serial dilutions and plating the diluted culture on agar plates.

3.5.1 Isolation of bacteria and resistance testing

Nutrient medium

A single colony of *Rhizobium trifoli* and *P. brassicacearum* was isolated by streaking a sterile inoculation loop from a primary active culture. The agar plate was incubated at 30^{o}C for four days. After four days, a single isolated colony of *Rhizobium trifolii* and *P. brassicacearum* was picked off using the sterilized loop and transferred into 100 ml of sterile nutrient broth (containing 1 g meat extract, 2 g yeast extract, 5 g peptone, 5 g NaCl, pH 7.4 at 37^{o}C purchased from Aldrich) and then incubated on a rotary shaker for 48 h.

250 ml Erlenmeyer flasks, each containing 100 ml of nutrient media were autoclaved, cooled, and amended with different concentrations (100, 200, 400, 600 and 800 mg Zn L^{-1}) of soluble $ZnSO_4$, and nanoparticles of ZnS and ZnO which were inoculated separately with the 48 h- old inocula prepared as explained above. Controls were made with media and inocula without added Zn.

Determination of growth

Growth was determined turbidimetrically by measuring the optical density of the experimental mixtures at 600 nm from 0 hour to 54 hours. The absorbance from controls (the media and inocula without the added Zn) was subtracted from the experimental value.

Growth was also determined by the dilution plate count method. All samples were incubated and shaken on the rotary shaker at room temperature (25^o to 28^{o}C) for 24 h. 1 ml of the solution was seven fold diluted with sterilized deionised water. 1 ml of the solution at 10^{-3} to 10^{-5} dilution from different concentrations (100 -800 mg L^{-1}) of $ZnSO_4$, ZnS and ZnO nanoparticles was applied uniformly on the surface of agar plates and incubated at 28^o C. Each plate was examined after 24- 48 h incubation. The lowest concentration of the study contaminant that inhibited growth was designated as the minimum inhibitory concentration. All the experiments were carried out in triplicate.

Plates that had between 30 and 300 colonies were counted and the viable cell count was calculated as follows:

Viable cell count (CFU/ml) =

$$\frac{Number\ of\ colonies \times dilution}{spreading\ volume\ (ml)} \qquad \text{(Equation 3.5)}$$

3.5.2 Hoagland solution

Bacteria resistance to Zn species in Hoagland solution was also investigated as future experiments with plants may be conducted hydroponically using Hoagland solution.

Isolation of the study bacteria and resistance testing followed the same method and procedures as described above. Hoagland solution prepared in the laboratory contained 1 ml of 1 M $NH_4H_2PO_4$, 6 ml of 1 M KNO_3, 4 ml of 1 M Ca (NO_3), 2 ml of 1 M $MgSO_4$, 1 ml of iron stock (Fe EDTA), and 1 ml mixture of 0.22 g $ZnSO_4$, 0.02 g H_2MoO_4, 0.08 g $CuSO_4$, 1.81 g $MnCl_2$, and 2.86 g H_3BO_3 (Hoagland and Arnon, 1950). Controls were Hoagland solution and inoculum without added Zn. Bacterial growth was assessed using the same methods as for the experiments with nutrient medium. Growth was determined turbidimetrically by measuring the optical density at 600 nm and colony forming units (CFU) were determined by the dilution plate count method.

3.6 Surface sterilisation of seeds

Sodium hypochlorite is the best choice for surface sterilisation (Barney, 2003) to eliminate contamination of microorganisms from seeds. Closed packets of *Brassica juncea* seeds were opened and allowed to equilibrate for 24 h at room temperature. Seeds were then rinsed for 5 mins in 5% NaClO, before rinsing thoroughly with sterile water and air drying in sterile cabinet.

3.6.1 Bacterial inoculation

Seed imbibement and soil inoculation have been frequently used for delivery, transport and distribution of bacteria (Huang et al., 2004; Germaine et al., 2009; Yousaf et al., 2010). The inoculation of seeds with plant growth promoting bacteria (PGPB) is known to increase plant growth (Verma et al., 2010; Adediran et al., 2015; 2016).

In this study, a seed inoculation method was used. A single colony of *Psuedomonas brassicacaerum* and *Rhizobium leguminosarum bv trifolii* was grown in a sterilised

nutrient broth and placed on a shaker at 30°C for 48 h. After the cultures reached an optical density at 600 nm ~1 (M501 Single Beam Scanning UV/ visible spectrophotometer), which corresponded to 10^9 CFU ml^{-1}, cells were collected by centrifugation at 3,000 g for 20 min at 4°C, washed with sterile water and re-suspended in sterile deionised water to a final concentration of 10^8 CFU ml^{-1}.

Sterilised seeds (as prepared above) were soaked in the bacteria suspension for 4 h and left standing in the laminar fume hood. Seeds for the control (without bacteria) treatment were also soaked in sterilised deionised water and maintained in the fume hood for 4 h.

3.7 Greenhouse pot experiments

The pot experiments were conducted in the greenhouse at the School of Biological Sciences, University of Edinburgh, in order to grow plants in regulated conditions such as light, ventilation, workspace availability, and temperature amongst others.
Pot experiments were conducted to assess the phytoextraction potential of *Brassica juncea (L.) Czern* and to assess its growth when elevated Zn concentrations are added to soil in the form of soluble Zn or as nanoparticulate ZnS and ZnO. Additional experiments assessed the effect of PGPB, *Rhizobium trifolii* and *Pseudomonas brassicacearum* on a number of plant growth and physiological parameters. Toxicity was evaluated by measuring (i) plant height, (ii) leaf count/size, (iii) dry biomass, and (iv) other observations such as leaf chlorosis, necrosis, drying and senescence. The general set-up of all pot experiments is described below and any specific details are explained in the later results and discussion chapters.

3.7.1 Experimental design

The experimental design varied in different chapters but mainly contained three different Zn treatments (Table 3.1) and controls in which *Brassica juncea* were grown with and without the presence of PGPB.

Table 3.1 Description of pot experiment design

Code	**Treatment**
Control	Control without Zn treatment
$ZnSO_4$	*Brassica juncea* grown on $ZnSO_4$ soil
ZnS nanoparticles	*Brassica juncea* grown on ZnS nanoparticles soil
ZnO nanoparticles	*Brassica juncea* grown on ZnO nanoparticles soil
B1 control	*Rhizobium leguminosarum* control without Zn treatment
B1 $ZnSO_4$	*Rhizobium leguminosarum* + *Brassica juncea* grown on $ZnSO_4$ soil
B1 ZnS nanoparticles	*Rhizobium leguminosarum + Brassica juncea* grown on ZnS NPs soil
B1 ZnO nanoparticles	*Rhizobium leguminosarum* + *Brassica juncea* grown on ZnO NPs soil
B2 control	*Pseudomonas brassicacearum* control without Zn treatment
B2 $ZnSO_4$	*Pseudomonas brassicacearum* + *Brassica juncea* grown on $ZnSO_4$ soil
B2 ZnS nanoparticles	*Pseudomonas brassicacearum + Brassica juncea* grown on ZnS NPs soil
B2 ZnO nanoparticles	*Pseudomonas brassicacearum* + *Brassica juncea* grown on ZnO NPs soil
sControl	Control soil no Zn treatment
s$ZnSO_4$	Soil treated with $ZnSO_4$

sZnS nanoparticles	Soil treated with ZnS NPs
sZnO nanoparticles	Soil treated with ZnO NPs

Each Zn treatment was replicated (three times) and the replicates were randomly distributed in the greenhouse (Figure 3.1).

Figure 3.1: Arrangement of pots spiked with 600 mg Zn kg^{-1} (soluble $ZnSO_4$, ZnS and ZnO nanoparticles), unspiked and control pots on a table in the greenhouse.

Following dose-response studies of *Rhizobium trifolii* and *Pseudomonas brassicacearum* on Zn species, one concentration of Zn (600 mg Zn kg^{-1}) was selected and used for pot experiments. The concentration selected was based on the Zn concentration that does not affect the viable bacteria count.

The air dried soil was weighed and amended with 600 mg Zn kg^{-1} of $ZnSO_4$, ZnS and ZnO nanoparticles in solid form. The spiked soil sample was mixed by hand for 1 hr to produce a homogenous mixture. 1 kg of spiked (Zn, ZnO and ZnS) or un-spiked soil (control) was placed in each 2.15 L pots. Pots were either planted or unplanted (pots containing spiked and unspiked soil without plants). The pots were placed in individual trays to capture drained leachate throughout the experiment and were left to equilibrate for a week in the greenhouse (Figure 3.1). Eight seeds were sown into both spiked and unspiked planted pots. Emergent seedlings were thinned out to a desired population density (3 plants per pot) at 12 days after planting. Pots were individually watered to maintain soil moisture during plant growth. The pot

experiment was conducted in a greenhouse set to provide a day/night temperature of 21 °C in a 18 h photoperiod at a photosynthetic photon flux density (PPFD) of 150 µmol m^{-2} s^{-1} provided by cool white fluorescent bulbs.

The growth parameters of *Brassica juncea (L.) Czern* were measured at different growing stages starting from seedlings. Plant height, from the soil surface to the tip of the longest shoot, was measured once a week on each replicate and for each treatment. The planted pots were harvested after 6 weeks of seed planting.

The unplanted pots also stood in the greenhouse to observe non-plant effects on soil Zn from 0 to 6 weeks.

3.7.2 Plant and soil sampling and analysis

All plants grown in both Zn spiked and unspiked soils were harvested after 6 weeks of growth. Shoots were cut 2 cm above the soil surface and washed with running tap water. Pots were emptied and roots were separated and washed in tap water to remove soil particles stuck on the root surface. The harvested plant material (roots and shoots) were dried in an oven to constant weight at 65^{o}C for 72 h. After cooling, dried samples were weighed to determine dry biomass. Dried samples were finely ground using a pestle and mortar and stored in polyethylene tubes prior to analysis.

Soil samples from different soil zones (bulk soil and rhizosphere) were homogenised separately before pH and total metal content analysis.

Dry-ash digestion of soil for total metal content

The dry ash method (Allen et al., 1974) was used for soil digestion. 5 g oven dried soil was weighed into weighed acid - washed crucibles and placed in a muffle furnace at 450^{o} C for 10 h. Soil samples were cooled in a dessicator and reweighed. 0.5 g of ashed material was weighed into crucibles, 5 ml concentrated HCl was added and covered with a watch glass and heated on a steam bath for 15 mins. 0.5 ml concentrated HNO_3 was added and heated for 2 h to evaporate to dryness. 1 ml

of HCl was added, swirled gradually to dissolve residue, and then diluted to 10 ml with deionised water and allowed to warm for 20 mins. The resulting digest was filtered through whatman No 44 filter paper and made up to 50 ml with deionised water. Blanks were prepared in the same way. The resulting digests were stored at 4^{o}C until analysis by ICP-OES (described in more detail in section 3.8).

Wet digestion for plant total Zn metal content

The wet digestion method (Allen et al., 1974) was used to prepare plant material for total metal content determination. 0.100g of finely ground, oven dried plant material was weighed into a Pyrex test tube. 2 ml concentrated H_2SO_4 was added and shaken to disperse the contents. 2 aliquots of 0.75 ml H_2O_2- 30% was added in a fume cupboard, properly mixed and allowed to stand. Test tubes were placed in a heating block (Grant BT5D) and heated to 320^{o}C for 6 h. The cooled clear solution was transferred to a 50 ml volumetric flask with washing and made up to 50 ml with deionised water. Blank digests were prepared in the same way and all samples and blanks were prepared in duplicate. The resulting digest were stored at 4^{o}C until analysis for Zn concentration by inductively coupled plasma – optical emission spectroscopy (Perkin Elmer Optima 5300 DV ICP-OES). Blanks were deducted from the analytical results. Both results were reported as the mean of two sub samples of each material.

3.7.3 Determination of phytoextraction and hyperaccumulation potential of *Brassica juncea (L.) Czern*

The resulting Zn concentrations from plant and soil analysis were used to evaluate Zn phytoextraction by *Brassica juncea* (L.) Czern. The following parameters were considered: bioaccumulation factor, translocation factor, tolerance index and phytoextraction efficiency, which are explained below.

Bioaccumulation factor (BAF) is the ratio of the concentration of metal in the plant tissue (root and shoot) to the initial metal concentration in soil (Zayed et al., 1998; Chopin and Alloway, 2007; Boussen et al., 2013; Ma et al., 2015).

$$BCF = C\ plant\ /\ C\ soil \quad \text{(Equation 3.6)}$$

This coefficient is effective in evaluating the metal accumulation by plants.

Translocation factor (TF) is used in evaluating the ability of plants to transfer metals to their aerial parts. TF is the ratio of the metal concentration in the shoot to the metal concentration in the root (Chopin et al., 2008; Sun et al., 2009; Ma et al., 2015).

$$TF = C\ shoot\ /\ C\ root \quad \text{(Equation 3.7)}$$

Tolerance index (TI) was also quantified (Yadev et al., 2009) as,

$$\frac{Mean\ height\ of\ the\ plant\ growing\ on\ contaminant\ soil\ \times 100}{Mean\ height\ of\ the\ plant\ growing\ without\ metal\ (control)} \quad \text{(Equation 3.8)}$$

Phytoextraction efficiency (PE) is defined as the ratio of an element accumulation in shoot to that in soil which is calculated as:

$$PE(\%) = \frac{Mshoot \times Wshoot}{Msoil\ \times Wsoil} \times 100 \quad \text{(Equation 3.9)}$$

Where M_{shoot} is the metal concentration in shoots of the plants (mg kg^{-1}), W_{shoot} is the plant dry above ground biomass (g), M_{soil} is the initial metal content in soil (mg kg^{-1}) and W_{soil} is the amount of soil in the pot (g). The PE values reflect the amount of a metal remediation by plant shoots from soil (Sun et al., 2011; Mani et al., 2015).

3.7.4 Estimation of chlorophyll content in the plant leaves

Chlorophyll is a photosynthetic pigment and measuring chlorophyll content is considered an important parameter for monitoring plant health (Sharma et al., 2014

Jiang et al., 2014; Cortazar et al., 2015). To examine if the Zn contaminated soil induced alterations in the photosynthetic pigments of *Brassica juncea (L.) Czern*, leaf chlorophyll content was determined according to the method of Arnon (1949). After plant harvest 0.5 g of fresh *Brassica juncea* leaves was weighed and torn into pieces. Torn leaves were ground and homogenised in a vortex for 30 s. Chlorophyll content was extracted in 2 ml of 80% acetone. The extract was transferred to centrifuge tubes and stored at 4°C in the dark for 24 h and then centrifuged at 3000 g for 15 min. Chlorophyll content was determined through spectrophotometric measurement at 663 and 645 nm (A_{663} and A_{645}, respectively) (Arnon, 1949; Nadler et al; 1972; Maitra et al., 2015; Tanwir et al., 2015). The total chlorophyll content was obtained by the addition of chlorophyll a and b values (Arnon, 1949; Chaurasia et al., 2013). Chlorophyll content was expressed as mg g^{-1} fresh weight, calculated as follows:

$$Chlorophyll\ a = 12.7\ (A_{663}) - 2.69\ (A_{645}) \qquad \text{(Equation 3.10)}$$

$$Chlorophyll\ b = 22.9\ (A_{645}) - 4.68\ (A_{663}) \qquad \text{(Equation 3.11)}$$

Total chlorophyll (mg g^{-1} FW) =

$$\left(20.2(A645) + 8.02\ (A663)\right) \times V \div 1000\ \times W \qquad \text{(Equation 3.12)}$$

Where W= weight of fresh plant (g) and
V= volume of extract in ml

3.7.5 Transmission electron microscopy to map Zn distribution in plant root samples

Transmission electron microscopy (TEM) has been used to ascertain metal accumulation in plants (Chen et al., 2007; Wilson and Bacic, 2012; Guzman et al., 2014). TEM was used in this research to map out the distribution pattern of Zn in

the roots of *Brassica juncea* plants. TEM is an important tool for studying plant structures. It operates on the same basic principle as the light microscope but uses focused electron beams as an illumination source and shorter wavelength that produces a resolution approximately a thousand times better than a light microscope (Fowke, 1995). Due its properties, the electron beam penetrates thin slices of plant material and permits the study of internal features of cells and organelles.

A transmission electron microscope fires a beam of electrons through a specimen to create a magnified image of an object. An electron gun at the top of a TEM emits electrons that travel through the microscope's vacuum tube. Instead of glass lenses focusing the light (as in the case of light microscope), the TEM uses electromagnetic lenses to focus the electrons into a very fine thin beam (Fultz and Howe, 2012). This thin beam then passes through the study specimen, and the image becomes visible when the electrons either scatter or hit a fluorescent screen at the bottom of the microscope (Reimer, 2013). This image can be viewed and studied directly (through a viewing portal) within the TEM or photographed with a camera (Figure 3.2).

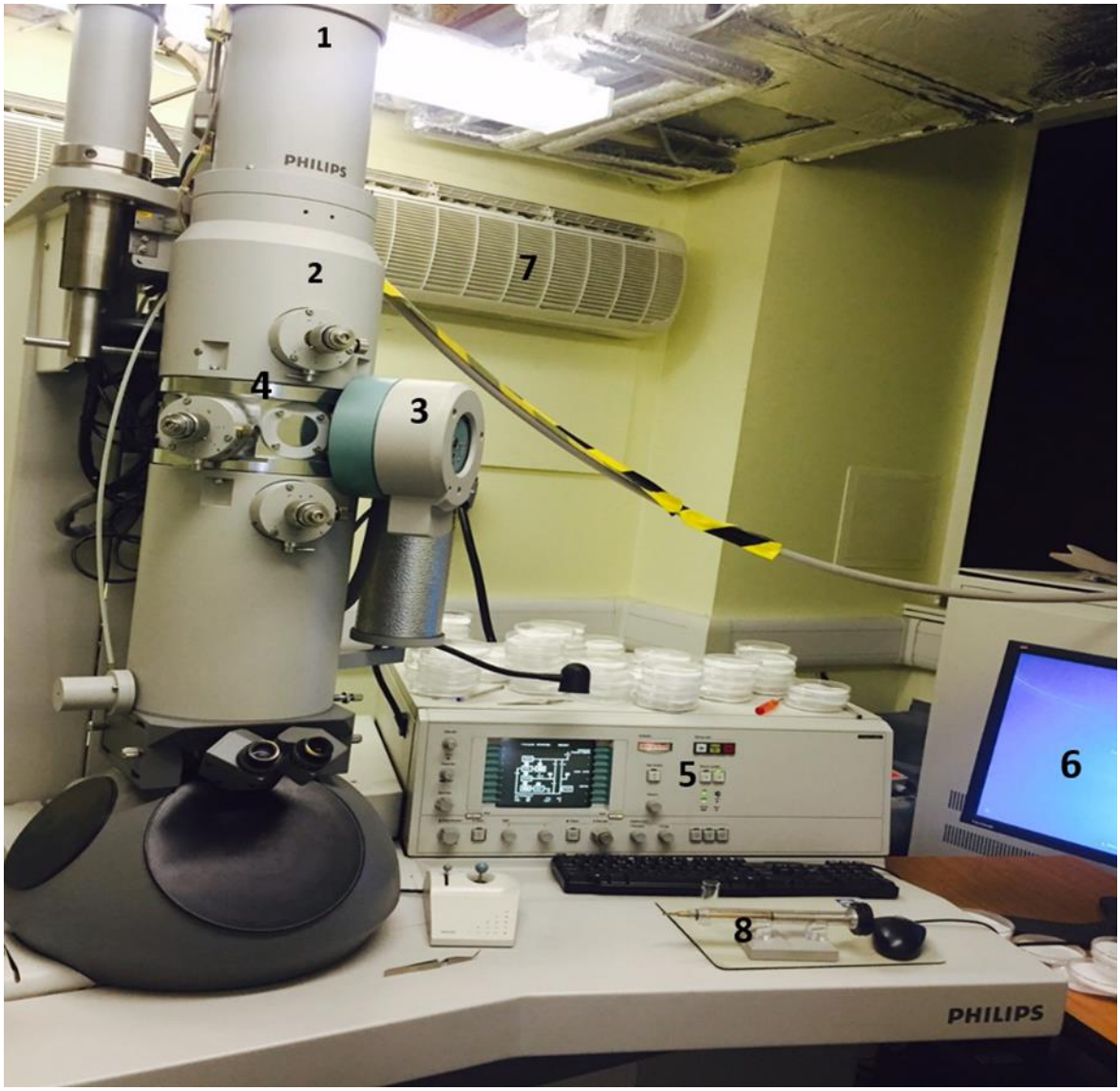


Figure 3.2: Philips CM120 transmission electron microscope used in the study.1: Electron cannon in the upper part of column. 2: Electromagnetic lenses to direct and focus the electron beam inside the column. 3: Opening for inserting samples. 4: Vacuum pump system. 5: Operation panel. 6: Image display. 7: Air conditioner for cooling the instrument. 8: Sample holder.

TEM Sample preparation and analysis

Roots of *Brassica juncea (L.)* Czern *from different* Zn treatments (Figure 3.3) were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 2 h then washed in three 10 minute changes of 0.1 M sodium cacodylate. Specimens were then post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate for 45 minutes, then washed in three 10 minute changes of 0.1 M sodium cacodylate buffer. These samples were then dehydrated in 50%, 70%, 90% and 100% normal grade acetones

for 10 minutes each, then for a further two 10-minute changes in Analar acetone. Samples were then embedded in agar 100 resin. Sections 1 µm thick were cut on a Reichert OMU4 ultramicrotome, (Leica Microsystems (UK) Ltd, Milton Keynes), stained with toluidine blue, and viewed in a light microscope to select suitable areas for investigation. Ultrathin sections, 60 nm thick were cut from selected areas, stained in uranyl acetate and lead citrate, then viewed in a Philips CM120 transmission electron microscope (University of Edinburgh, UK). Images were taken on a Gatan Orius CCD camera.

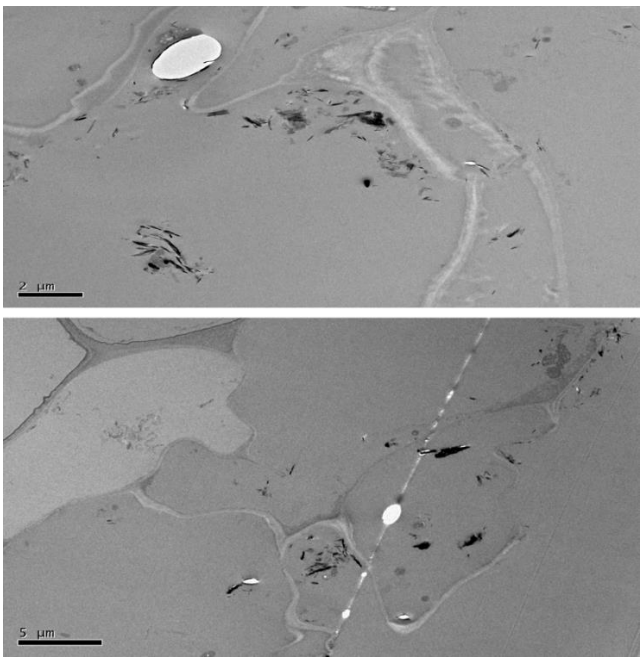


Figure 3.3: TEM micrographs of (2 and 5 µm) magnifications image of *Brassica juncea* (L.) roots exposed to 600 mg Zn kg^{-1} $ZnSO_4$.

3.8 Inductively coupled plasma-optical emission spectrometry (ICP-OES)

Elemental concentrations were determined in the plant and soil samples using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES). ICP-OES has emerged as useful technique for trace metal analysis of soil owing to its high detection power, low sample consumption and multielement capability. ICP-OES works on the principle that when atoms and ions are excited, light is emitted. The wavelength and intensity of the light reflects the elements present in the sample (Moore, 1989). In using the ICP-OES, the solution to analyse is aspirated by a peristaltic pump which delivers the dissolved sample into an analytical nebulizer

where it is transformed into an aerosol. The aerosol produced is directly led into a very hot (10000^0 K) radio frequency argon plasma by strong magnetic field. The elements in the samples become excited and the electrons emit energy at a characteristic wavelength as they return to ground state. The emitted light is then measured by optical spectrometry. The main disadvantage of ICP-OES is that the solid sample is required to be in solution prior to the determination of metal content (Sastre et al., 2002).

A Perkin Elmer Optima 5300 DV ICP Emission Spectrometer at the University of Edinburgh, UK was used for the ICP-OES analyses. The operating conditions and wavelengths are listed in Tables 3.2. and 3.3.

Table 3.2 Instrumental parameters for trace element determination.

Nebulizer gas flow rate	0.75 dm^3 min^{-1}
Auxillary gas flow	0.2 dm^3 min^{-1}
Plasma gas flow	15 dm^3 min^{-1}
Auto sampler	1.5 cm^3 min^{-1}
ICP RF power	1400 W
Wavelength range	160 -782 nm
Spray chamber type	Scotts Spray Chamber
Plasma viewing	Axial / radial
Rinse delay	30 s
Rinse solution	2 % v/v HNO_3

Table 3.3: Wavelengths used for the analysis of individual analytes

Analyte	Wavelength (nm)
Ca	317.933 /393.366
Fe	259.939
K	766.498
Mg	285.213
Mn	259.372
Na	589.6
P	178.223
S	180.671
Zn	202.548 / 334.501

Multi-element calibration standards (0 to 1000 ppb of Ca, Fe, K, Mg, Mn, Na, P, S and Zn) were mixed from single element stock standard solutions. All calibration standards were prepared in a matrix of 2 % v/v HNO_3. The calibration standards required an R^2 value of at least 0.9999 in order to present a satisfactory calibration curve. Quality control blank checks and external calibration verification checks were run regularly throughout the analysis. An external standard (Merck ICP Multi element standard solution VI CertiPUR®) was analysed at different dilutions as a cross reference for the calibration graphs. Data acquisition and analysis was completed using Perkin Elmer WinLab ™ ICP operating software (Perkin Elmer, 1997).

To determine total element concentrations in soil and plant samples in mg kg^{-1}, the ICP-OES concentration results in mg L^{-1} were corrected for the dry weight of sample and volume used in the extraction procedure. Blanks from the same extraction procedure were also analysed and mean values subtracted from the analytical result.

Precision of analysis can be expressed by the relative standard deviation (RSD) or percent relative standard deviation, calculated from the mean of replicated samples (Chen and Ma, 2001) as;

Precision (%) =

$$[(standard\ deviation\ of\ means) \div means] \times 100 \qquad \text{(Equation 3.13)}$$

3.9 Soil pore water extraction and analysis

The concentration and speciation of metals in soil pore water may provide more useful information on metal bioavailability and toxicity than total soil concentration (Hani, 1996; Percival, 2003; Prokop *et al*., 2003; Shan *et al*., 2003). The soil pore water composition represents the natural medium for plant growth and allows the prediction of plant responses to metal in the soil environment (Di Bonito et al., 2008). Centrifugation has been widely applied to extract pore waters from various materials including sediments, chalks, sandstones and clayey soils (Shaffer et al., 1937; Richards and Weaver, 1944). Centrifugation of soil pore water is used to fractionate pore water by selecting centrifugation rates (relative centrifugal force (RCF) value and centrifugal speed). Increasing the centrifugal speed and therefore the relative centrifugal force during soil centrifugation, release less available water (Tyler, 2000).

Soil pore waters were extracted by centrifugation (Krishnamurti et al., 2013; Ma et al., 2013) of 150 g of Zn spiked soil ($ZnSO_4$, ZnS and ZnO nanoparticles) at the start of experiment (0 week) and at 6 weeks. Soil samples from the 600 mg Zn kg^{-1} unplanted pots (two duplicates from each treatment) were placed in a centrifuge tube. Centrifugation was performed using a Sorvall™ RC 6 plus centrifuge (Thermo scientific) at 12,000 g for 1 h at 4° C and the sample further passed through 45-µm Millipore filters. The concentration of Zn in the soil pore water was determined by ICP-OES.

3.10 X-Ray Absorption Spectroscopy (XAS) studies on soil and plant roots

X-ray absorption fine structure (XAFS) was used in this study to investigate the distribution and forms of Zn in plants and soil. This method uses X-rays to provide information on the co-ordination environment of the element of interest. The basic principle of XAFS arises from the excitation of a core level electron of the absorbing atom using x-rays of sufficient energy. The XAFS spectrum of an element in a sample is generated using an intense synchrotron X-ray source with a broad continuous range of energies. In the synchrotron, electrons are accelerated and directed into storage rings which has auxiliary components like bending magnets and insertion devices (undulators or wrigglers) needed to convert high energy electrons to light (Calas et al., 1987; Brown et al., 1998; Brown and Sturchio, 2002). XAS uses two energy regions to examine various coordination properties of the element of interest. The energy region from 30 eV to 800 eV above the edge is known as the EXAFS (extended x-rays absorption fine structure) and from below 30 eV below to 100 eV above the absorption edge it is known as the XANES (x-ray absorption near edge structure) (Durham 1988). XAS measures the energy dependence of the X-ray absorption co-efficient μ (E) at and above the absorption edge of a selected element. X-ray absorption can be measured in two ways:

Transmission: the absorption is measured directly by measuring what is transmitted through the sample: $\mu(E)x = \log (I_0 / I_t)$

*Fluorescence***:** the electron emitted is measured as: $\mu(E)x \sim I_f / I_0$

Where I_0 is the X-ray intensity hitting the material, I_t is the intensity transmitted through the material, x is the thickness of the material or absorber and I_f is the monitored intensity of a fluorescence line (electron emission) associated with the absorption process.

Beamlines I18 and B18 at the Diamond Light Source UK were used in this research for Zn speciation in soil and plants samples. The different beamlines were used depending on the type of data required (e.g. I18 was used for mapping of Zn hotspot in plant roots). The key features of the I18 microfocus spectroscopy

beamline at the Diamond Light Source, UK are described by Mosselmans et al. (2009) and are shown in Figure 3.4.

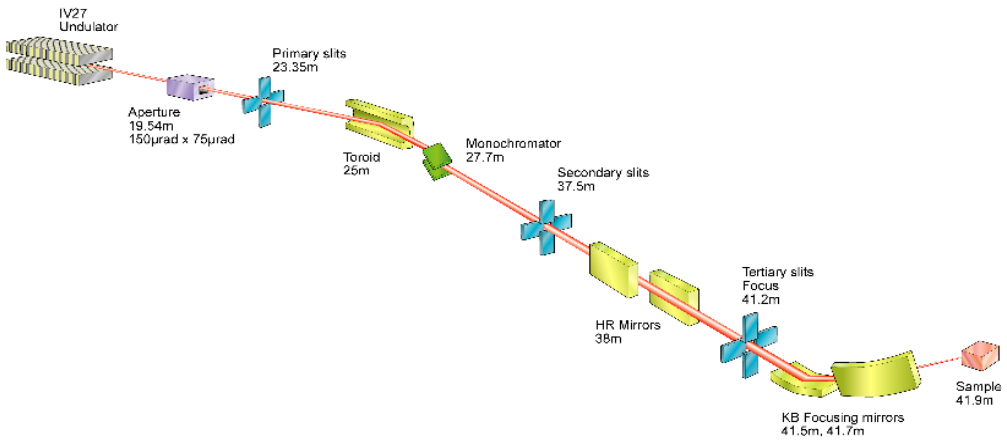


Figure 3.4: A schematic view of the principal optical elements of the beamline I18. Photons from the storage rings are delivered *via* the monochromator and a slit system to the samples. Figure from Mosselmans et al. (2009).

3.10.1 X-ray absorption spectroscopy (XAS) on beamline I18

A separate pot experiment was conducted for X-ray absorption spectroscopy analysis. The same method described already for growth experiments was followed, which also provided replicate plant growth data. However, due to the long time required to analyse each sample on beamline I18 and in order to increase the X-ray signal only 600 mg Zn kg^{-1} plant samples were analysed. Live plants were transported for mapping and X-ray absorption spectroscopy (XAS), XANES and EXAFS analyses on microfocus beamline I18 at the Diamond Light source UK.

Sample preparations.

After harvest, fresh roots and stems of *Brassica juncea* plants grown in soil treated with 600 mg Zn kg^{-1} of $ZnSO_4$, ZnO and ZnS nanoparticles were washed thoroughly with deionised water to remove any surface contaminants. Roots and stems were cut with a scalpel and embedded in Meta-mix for eight hours. The solidified root and stem samples were axially sectioned (30 µm) using a Reichert Ultracut microtome. The sample was placed on a sapphire disc, covered with kapton tape and loaded into an aluminium sample holder, which was cryofixed with liquid nitrogen and inclined at an angle of 45^o to the incident beam (Figure 3.5).

Zinc distribution in roots and stems were mapped with incident energy of 10.5 k_ev. The energy was scanned through the absorption edge of Zn (9630 – 9850 eV) and was calibrated by recording the absorption edge of Zn foil. The X-ray absorption spectrum was collected in fluorescence mode on the microfocus beamline I18 using a nine- element ORTEC germanium solid state detector. The detector was placed in the horizontal plane at right angles to the beam axis in order to reduce detection of elastically scattered photons. XAFS mapping was performed at 0.5 x 0.5 mm with 2 µm resolution. After the X, Y scan was done several hot-spots were identified. XAFS data were collected at the Zn K-edge from regions of high Zn content. Depending on Zn concentration, 5- 20 scans of 30 minutes each were recorded and averaged at each spot analysed. These high Zn regions were selected for collection of µ-XANES spectra.

Zn K-edge µ-XANES spectra were also collected under similar beam conditions for selected Zn model compounds (Terazan et al., 2008). Model compounds were analysed in transmission mode.

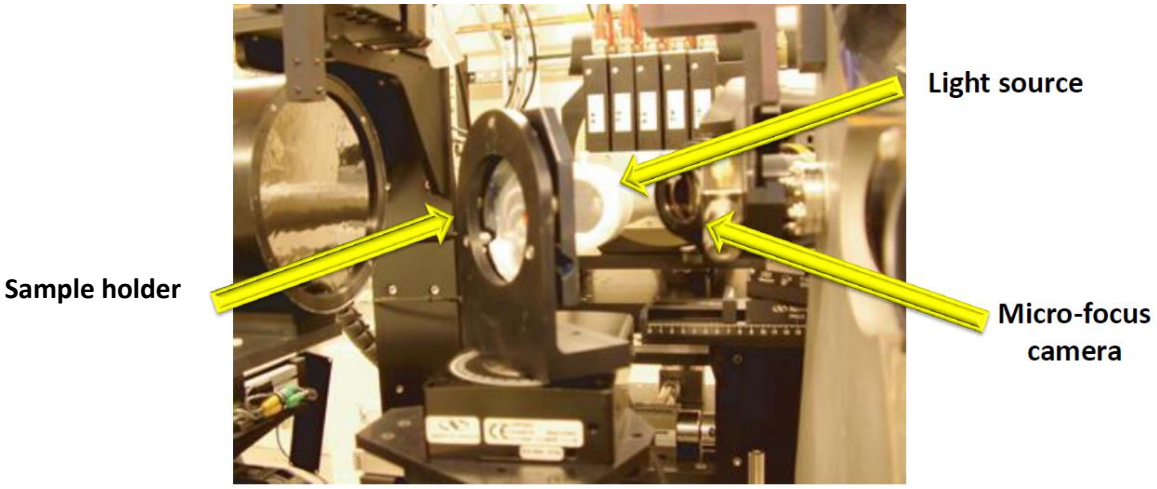


Figure 3.5: Beamline I18.

For this study, the following samples were analysed as seen in Table 3.4.

Table 3.4: Experimental samples analysed by XAFS on beamline I18

Plant samples	Roots	Root + *Pseudomonas brassicacearum*	Shoot
$ZnSO_4$	2	1	1
ZnO	2	1	1
ZnS	2	1	1

Due to the allotted short beam time, this study focused more on the roots as roots are the main plant organ with an enormous surface area that absorbs and accumulates contaminants and interacts with soil bacteria (Fitters, 1987; Zhou et al., 2013) rather than shoots.

3.10.2 X-ray absorption spectroscopy (XAS) on beamline B18

A separate pot experiment was also conducted for X-ray absorption spectroscopy focussing on Zn speciation in different soil fractions. Changes in Zn speciation between bulk soil, root-plant interface soil (the rhizosphere) and root tissue of *Brassica juncea* grown in 600 mg Zn kg^{-1} soil (with and without plant growth

promoting bacteria) was examined using Beamline B18 at the Diamond Light Source, Didcot, UK.

Duplicate samples from roots, bulk and rhizosphere soil were freeze dried prior to XAS analysis. ~40 mg bulk and rhizosphere soil from 600 mg Zn kg^{-1} soil treated with $ZnSO_4$ and ZnS nanoparticles were finely ground and mixed with 150 mg cellulose into pellets of ~ 1mm thickness under a pressure of 2 tonnes to optimise the effective edge- step of the XAFS data. Freeze dried plant roots were also finely ground. Root samples were placed on a sapphire disc, covered with kapton tape and loaded into a Perspex sample holder, inclined at an angle of 45^{o} to the incident beam which was cryofixed with liquid nitrogen. Beamline B18 was operating in qexafs mode setup with a fast scanning Si (111) double crystal monochromator which was calibrated using Zn foil edge. Data was collected in fluorescence mode using a 36- element Ge solid state detector. On average 10 scans (5 mins) were acquired to improve the signal to noise ratio.

XAS standards were also finely ground and ~15 mg mixed with 150 mg cellulose into pellets to optimise the effective edge-step of the absorption edge. XAS data for Zn citrate, Zn carbonate, Zn acetate and Zn sulphate standards were also collected at the Zn edges in transmission mode with the ion chamber filled with inert gas to optimize sensitivity. XAS data for the remaining Zn model compounds were collected in fluorescence mode. Data were also recorded simultaneously from a reference Zn foil in a third chamber. For this study, the following samples were analysed as seen in Table 3.5.

Table 3.5: Experimental samples analysed by XAFS on beamline B18

Plant samples	Roots	Rhizosoil	Bulk soil
$ZnSO_4$	2 with *Rhizobium leguminosarum* + 1 without bacteria	2 with *Rhizobium leguminosarum* + 1 without bacteria	2 with *Rhizobium leguminosarum* + 1 without bacteria
ZnS nanoparticles	2 with *Rhizobium leguminosarum* + 1 without bacteria	2 with *Rhizobium leguminosarum* + 1 without bacteria	2 with *Rhizobium leguminosarum* + 1 without bacteria

3.10.3 Synthesis of Zn reference compounds

The reference compounds shown in Table 3.6 were freshly prepared following standard protocols designed for XAS analysis (Terazno et al., 2008). 7 mM of Zn nitrate solution was mixed with a solution of each organic compound (70 mM oxalate, phytic acid, histidine, cysteine, phosphate, polygalacturonate, formate). The pH of the reference solutions was adjusted to 7 by adding 1M NaOH.

Table 3.6: Zinc reference compounds analysed for the XAS studies

Zn standard compounds	Characteristics
Zn oxalate	7.0 mM $Zn(NO_3)_2$ + 70 mM sodium oxalate, pH 7.0
Zn phosphate	7.0 mM $Zn(NO_3)_2$ + 70 mM sodium phosphate, pH 7.0
Zn histidine	7.0 mM $Zn(NO_3)_2$ + 80 mM Histidine, pH 7.0
Zn cysteine	7.0 mM $Zn(NO_3)_2$ + 70 mM cysteine, pH 7.0
Zn phytate	7.0 mM $Zn(NO_3)_2$ + 70 mM phytic acid solution, pH 7.0
Zn polygalacturonate	7.0 mM $Zn(NO_3)_2$ + 70 mM polygalacturonic acid solution, pH 7.0
Zn formate	7.0 mM $Zn(NO_3)_2$ + 70 mM formic acid solution, pH 7.0

Zn sulphide NPs	Synthesised in the laboratory

Zn citrate, carbonate, acetate, nitrate and sulphate were purchased from Sigma Aldrich and also included as reference materials. The Zn solid standards were finely ground in a pestle and mortar and 15 mg mixed with 150 mg cellulose and made into pellets whilst the solutions were stored in polyethylene falcon tubes prior to XAS analysis. All these Zn references compounds except ZnO nanoparticles were freshly prepared and used in the XAS study on Beamline B18.

3.10.4 Visualisation and linear combination modelling

The XRF spectra collected in Beamline I18 were analysed (including background subtraction and peak fitting) using PyMCA 4.4.1 software (Sole et al., 2007). XANES spectra in both studies represent a combination of all Zn species present in sample transected by the beam. In order to assess chemical species information, LCF (linear combination fitting) was used to identify the relative proportions of reference spectra within the data for samples of root, bulk and rhizosphere soil (B18) and roots (I18). The resulting Zn speciation in each sample is dependent on the suite of reference spectra available to fit the sample spectra. Athena from IFFEFIT software package (Ravel and Newville 2005) was used to conduct LCF on the XANES data collected from B18 and I18.

LCF is a way of calculating the contribution of a range of reference (standards) compounds run under identical conditions to XANES signal of the sample. The goodness of the fit was estimated by determining the residual R factor of the fit (Langner et al., 2012).

$$R = \frac{\sum(data-fit)^2}{\sum(data)^2} \qquad (Equation\ 3.14)$$

A lower R factor represents the best fit between the sample spectrum and the fitted standard spectra (Hettiarachchi et al., 2006; Terzano et al., 2008).

3.11 Conclusion

The general experimental and theoretical techniques have been described in this chapter. This included the materials, methods and data analyses employed and the justification of their use purpose. The next chapters of this thesis are based on the results obtained from experiments conducted to address the research questions previously defined.

Chapter 4

4 Testing *Rhizobium leguminosarum* and *Pseudomonas brassicacaerum* resistance to Zn and toxicity of different Zn species to *Brassica juncea*

4.1 Introduction

Particles with novel and distinctive physicochemical properties with at least one dimension within the range of 1- 100 nm are defined as nanoparticles (NPs) (Moore, 2006). Zn minerals occur naturally as bulk soilds but also in the form of nanoparticles such as ZnS (Moreau et al., 2007). ZnO and ZnS NPs are also used in a wide range of products such as textiles, sunscreens, paints, industrial coatings, electronic and optoelectronic applications (solar cells, ultra violet (UV) lasers, infrared windows, sensors, electroluminescence devices) (Hernandez-Viezcas et al., 2011; Li et al., 2013; Sakshi et al., 2013; Thottoli and Unni, 2013). Following their production, transportation, and usage, these Zn species are released into the environment intentionally or accidentally (Zhang et al., 2007; Wu et al., 2010; Dhas et al., 2013; Feng et al., 2013). NPs can be toxic to terrestrial organisms (Morones et al., 2005; Griffith et al., 2007; Lin and Xing, 2007; Roberts et al., 2007; Lopez-Moreno et al., 2010). ZnO NPs at a concentration between 3 and 10 mM were shown to cause 100 % inhibition of bacteria growth (Brayner et al., 2006).

Metal toxicity to bacteria has been investigated using either bacteria as a single isolate, in solution, soil, water, or a mixed culture with varying metal concentrations (Richards et al., 2002; Rathnayake et al., 2013). The selection of culture media is important when investigating metal toxicity to bacteria (Richards et al., 2002; Rathnayake et al., 2013). High levels of metal tolerance by bacteria have been demonstrated when grown in culture media with a defined chemical composition and containing a high concentration 2-10 g L^{-1} of a carbon source (e.g., mannitol, gluconate, glucose) (Rathnayake et al., 2013).

Upon metal exposure, some bacteria are resistant or tolerant to extremely high concentrations of metal (Trevor et al., 1985). Terms such as “resistance” or “tolerance” are often used interchangeably in the literature, although Gadd (1992)

distinguishes between them as follows. 'Resistance' is defined as the ability of bacteria to survive toxic effects of metal exposure by means of a detoxification mechanism, whilst 'tolerance' is the ability of bacteria to survive high concentrations of a metal by means of intrinsic properties (e.g. extracellular polysaccharide and metabolite excretion, pigmented cell walls) (Gadd, 1992).

Plant growth promoting bacteria (PGPB) tolerate high concentrations of metal contaminants which may be significant in the restoration of contaminated soil (Krishna et al., 2013). Information on the toxicity of different Zn species to PGPB and hyperaccumulating species is required to design phytoremediation experiments, and ultimately to design sustainable phytoremediation treatments of Zn contaminated soils. Hyperaccumulating species such as *Brassica juncea (L.) Czern* are amongst the Brassica species that are widely used in phytoremediation of metal contaminated soil due to their ability to tolerate, extract and accumulate metals (Purakayastha et al., 2008; Dede et al., 2012). Thus, it is important to evaluate the effect of different Zn species on *Brassica juncea,* including germination and early seedling growth.

This study focuses on: (a) the resistance of *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* to Zn species; (b) the phytotoxicity of different Zn species on *Brassica juncea*; and (c) the role of *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* on germination and early seedling growth of *Brassica juncea.*

It was hypothesised that:

(i) *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* will be resistant to Zn.

(ii) *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* will confer Zn tolerance effect, leading to improved plant growth than in uninoculated plant under Zn contamination.

4.2 Materials and methods

4.2.1 Materials

This study focuses on the toxicity of Zn in nanoparticulate form by comparing Zn from Zn sulfate solution with the Zn NPs suspensions. Suspensions of ZnO and ZnS NPs and Zn sulfate solution were used as the source of zinc contamination in this study. Zinc sulfate ($ZnSO_4.7H_2O$) and ZnO nanoparticles with a nominal particle size of 35 ± 5 nm were purchased from Sigma Aldrich UK. A stock solution of zinc sulfate was sterilised by filtration with 0.22 µm Millipore sterilized filters. ZnS nanoparticles were synthesized in the laboratory (described in Chapter 3). ZnS and ZnO nanoparticles were suspended directly in deionized water and dispersed by sonication (Decon Fs 200b sonicator, 30^oC) for 1 hr using the procedure of Lin and Xing, (2007). Small magnetic bars in the suspensions were used for stirring to avoid aggregation of particles prior to use. The pH value of the suspensions ranged from 6.2 to 7.3.

4.2.2 Micro-organisms

The bacterial strains used in this study were *Rhizobium leguminosarum* and *Pseudomonas brassicacearum.* These plant growth promoting bacteria are known to colonise plant roots, enhance Zn accumulation in plant and have the ability to promote plant growth (Adediran et al., 2015; 2016). *Pseudomonas brassicacearum subsp. brassicacearum* (strain DBK11*)* was originally isolated from the rhizoplane of a Brassica plant (Ortet et al., 2011; Loewen et al., 2014) and was obtained as a lyophilisate (DSM number 13227) from the German collection of microorganisms and cell cultures (Leibniz Institute, DSMZ). Frozen strains of *Rhizobium leguminosarum (bv) trifolii (strain WSM 1325*), originally isolated from red clover nodules, were obtained from the culture collection of the Ashworth Laboratory, School of Biological Sciences, The University of Edinburgh, UK.

4.2.3 Isolation of bacteria and resistance testing

a) Nutrient medium

Isolation of study bacteria and resistant testing followed the same method and procedures as described in Chapter 3.

(b) Hoagland solution

Since as part of this research, it was intended to conduct experiments with plants hydroponically in Hoagland solution, the toxicity of different Zn species to bacteria was also investigated in Hoagland solution. Isolation of study bacteria and resistant testing followed the same method and procedures as described in Chapter 3. The measured pH of the Hoagland solution was pH 5.6. Bacteria growth was determined turbidimetrically by measuring the optical density of the experimental mixtures at 600 nm from the starting time (0 h to 54 h). The dilution plate count method was used to assess the viability of the bacteria by determining the colony forming units (CFU). See detailed description of determination of bacteria growth in Chapter 3.

4.2.4 Seed inoculation and germination

A single colony of *Psuedomonas brassicacearum* and *Rhizobium leguminosarum bv trifolii* were grown in a sterilised nutrient broth (containing 1 g meat extract, 2 g yeast extract, 5 g peptone, 5 g NaCl, pH 7.4 at 37^{o}C purchased from Aldrich) and placed on a shaker at 30^{o}C for 48 hrs. Cells were collected by centrifugation at 3,000 rpm for 20 min at 4^{o}C, then washed thrice with sterile water and re-suspended in sterile deionised water to a final concentration of 10^{8} CFU mL^{-1} at 30^{o}C. Seeds of *Brassica juncea* were purchased (Sow Seeds Ltd., UK) and stored in a clean plastic bag in the dark at room temperature (14-16 ^{o}C) until use. Seeds were surface sterilised just before use in 5% sodium hypochlorite for 5 mins and rinsed three times in sterile deionised water in a laminar fume hood. Sterilised seed were immersed in 10 ml bacteria suspension for 4 h standing in the fume hood. Seeds for control treatments were also soaked in sterilised deionised water and maintained in the fume hood for 4 h.

Germination tests were carried out in petri dishes (9 cm diameter). 5 mL of different $ZnSO_4$, ZnS and ZnO nanoparticles were added, all at 600 mg L^{-1} Zn concentration, to the autoclaved Whatman no. 1 filter paper. This Zn concentration was chosen because it is high enough to stimulate undesirable effects on plants (Rico et al., 2011; Zhao et al., 2013) so the effectiveness of PGPBs inoculated treatments could be tested for ameliorating Zn toxicity to plants. Deionised water was used for the control treatments. 10 seeds were placed, spaced out, on top of the lower filter paper in the dish. The petri dishes were sealed with adhesive tape (Parafilm^{TM}) to avoid desiccation. All experiments and controls were conducted in triplicate (Table 4.1). The petri dishes were placed in a growth room at a constant temperature of 21^{o}C for germination to occur. Fluorescent lighting provided a photoperiod of 16 h d^{-1}. Seed germination was recorded after 2 days of seed sowing and re-sealed with adhesive tape. Seedlings were allowed to grow in the sealed petri dishes for 14 days and their growth measured.

Table 4.1 Description of experimental treatments

Codes	**Treatments**
Control	Control without Zn treatment
$ZnSO_4$	*B. Juncea* grown on $ZnSO_4$ solution
ZnS NPs	*B. Juncea* grown on ZnS NPs solution
ZnO NPs	*B. Juncea* grown on ZnO NPs solution
B1 Control	*R. leguminosarum* control without Zn treatment
B1 $ZnSO_4$	*R. leguminosarum* + *B. Juncea* grown on $ZnSO_4$ solution
B1 ZnS NPs	*R. leguminosarum* + *B. Juncea* grown on ZnS NPs solution
B1 ZnO NPs	*R. leguminosarum* + *B. Juncea* grown on ZnO NPs *solution*
B2 Control	*P.brassicacearum* Control without Zn treatment
B2 $ZnSO_4$	*P.brassicacearum* + *B. Juncea* grown on $ZnSO_4$ solution
B2 ZnS NPs	*P.brassicacearum + B. Juncea* grown on ZnS NPs *solution*
B2 ZnO NPs	*P.brassicacearum* + *B. Juncea* grown on ZnO NPs *solution*

Seed germination percentage was calculated for each petri dish according to the International Seed Testing Association method (ISTA, 2008). A seed was considered germinated after radicle emergence. Germination percentage was calculated as,

$$Germination\ (\%) = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds} \times 100 \quad \text{(Equation 4.2)}$$

Seedling shoot and root length was measured after 14 days with a ruler.

The tolerance index (TI) of a plant to Zn was calculated (Yadev et al., 2009) using a plant growth parameter, such as shoot and root length, as:

$$TI = \frac{Mean\ length\ of\ plant\ growing\ in\ contaminated\ media}{Mean\ length\ of\ plant\ growing\ in\ uncontaminated\ media\ (control)} \times 100 \text{ (Equation 4.3)}$$

4.3 Statistical analysis

All experiments were carried out in triplicate. The mean and standard error of mean of the three replicates of each Zn treatment, including the inoculated treatments were performed on Microsoft Excel software 2013. All treatment means were normally distributed using the Anderson-Darling' normality test. Root and shoot length were compared between treatments using two way analysis of variance (ANOVA). Tukey's HSD test was used to identify significant differences between treatment means. Anderson Darling' normality test and two-way analysis of variance were conducted using Minitab software version 17 (Minitab ™ Inc, State College, PA, USA).

4.4 Results

4.4.1 Effect of Zn addition on growth curves

The dose response experiment assessed the toxicity of the Zn contaminants in the medium on *Pseudomonas brassicacearum* and *Rhizobium leguminosarum.* Since, both study bacteria exhibited similar growth patterns in the different Zn treatments results, *Pseudomonas brassicacearum* only are reported here. The experimental results displayed in Figure 4.1 showed a marked difference in bacteria growth between $ZnSO_4$ and control as reflected by the optical density. *Pseudomonas brassicacearum* practically stopped growing upon increasing concentration of $ZnSO_4$ compared with the control, even at 100 mg L^{-1}. As a function of time, compared to $ZnSO_4$, *Pseudomonas brassicacearum* exhibited a different growth response to ZnO NPs. As concentration increased, *Pseudomonas brassicacearum* showed a trend of increasing from 100 – 200 mg L^{-1} whereas, at 600 – 800 mg L^{-1} there was also reduction in growth rate as compared with the control. The dose response to ZnS NPs was similar to the typical bacteria growth curve, as *Pseudomonas brassicacearum* increased steadily over the 54 h experimental period. Growth in the Zn- amended treatments declined after 40 h as compared with the control in which growth did not decline until after 52 h. Growth rates decreased with increasing ZnS concentration, although growth occurred at all concentration tested.

The dose response results of the three study contaminants as reflected by the optical density assessment of bacteria growth revealed that $ZnSO_4$ was the most toxic since it impaired bacterial growth from the lowest concentration to highest concentration. The order of toxicity of the study contaminants to the growth of *Pseudomonas brassicacearum* was $ZnSO_4$ > ZnO NPs > ZnS NPs.

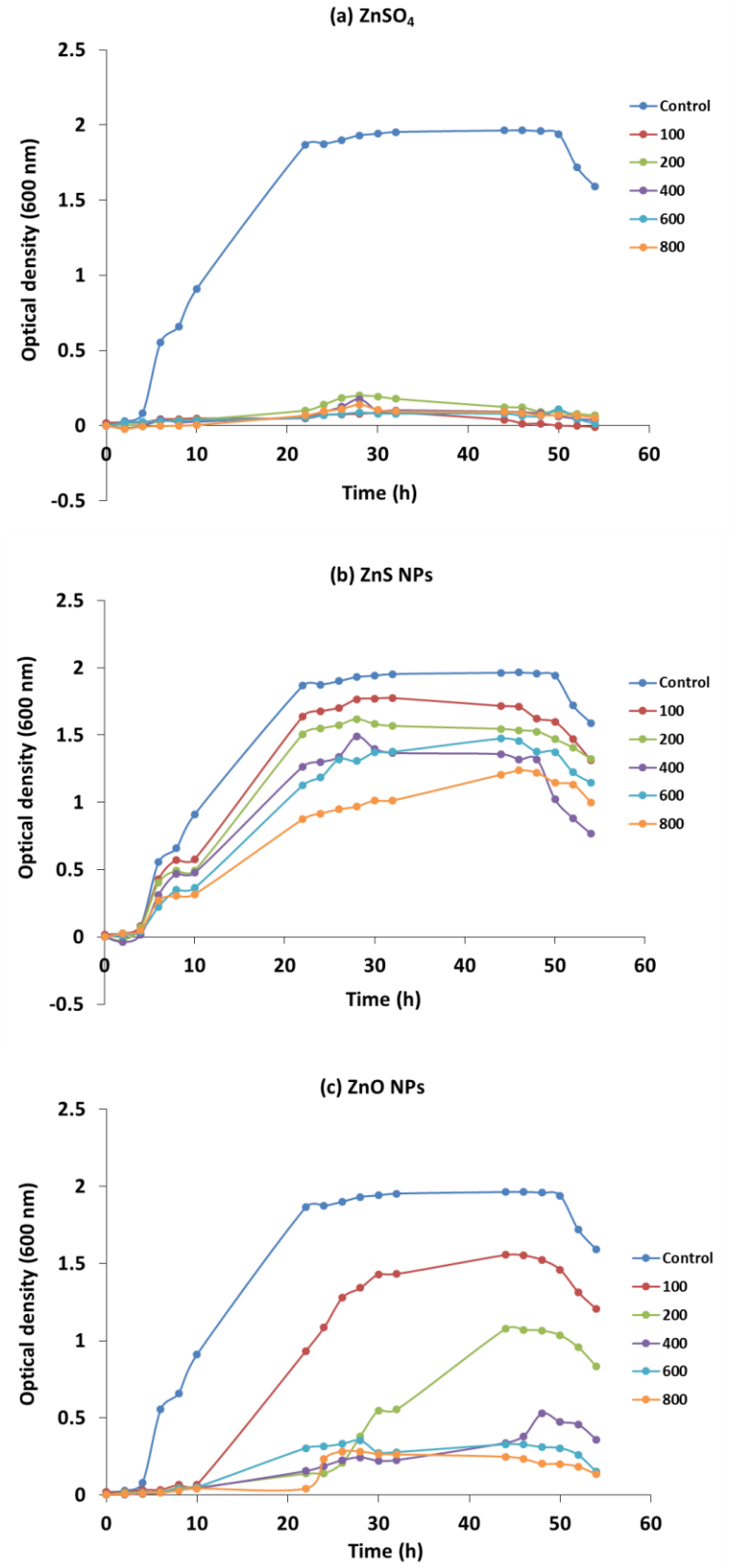


Figure 4.1: Growth of *Pseudomonas brassicacearum* in uncontaminated nutrient broth and nutrient broth amended with 100 – 800 mg L^{-1} (a) $ZnSO_4$ (b) ZnS nanoparticles (c) ZnO nanoparticles. Since nanoparticle suspensions contributed

to light scattering (absorption) at 600 nm, the absorbance measured at time zero was subtracted from all measurements for nanoparticle suspensions.

To compare with the results from the nutrient broth experiments in Figure 4.1, the growth curves of *Pseudomonas brassicacearum* for experiments conducted in Hoagland media are shown in Figure 4.2. Although growth occurred in the controls, the optical measurements in the flasks containing Zn were compounded by the precipitation of Zn hydroxides, resulting in apparent negative growth in most of experiments. Consequently, growth in Hoagland solution could not be reliably assessed and plans to use this medium in plant growth experiments in this project were abandoned.

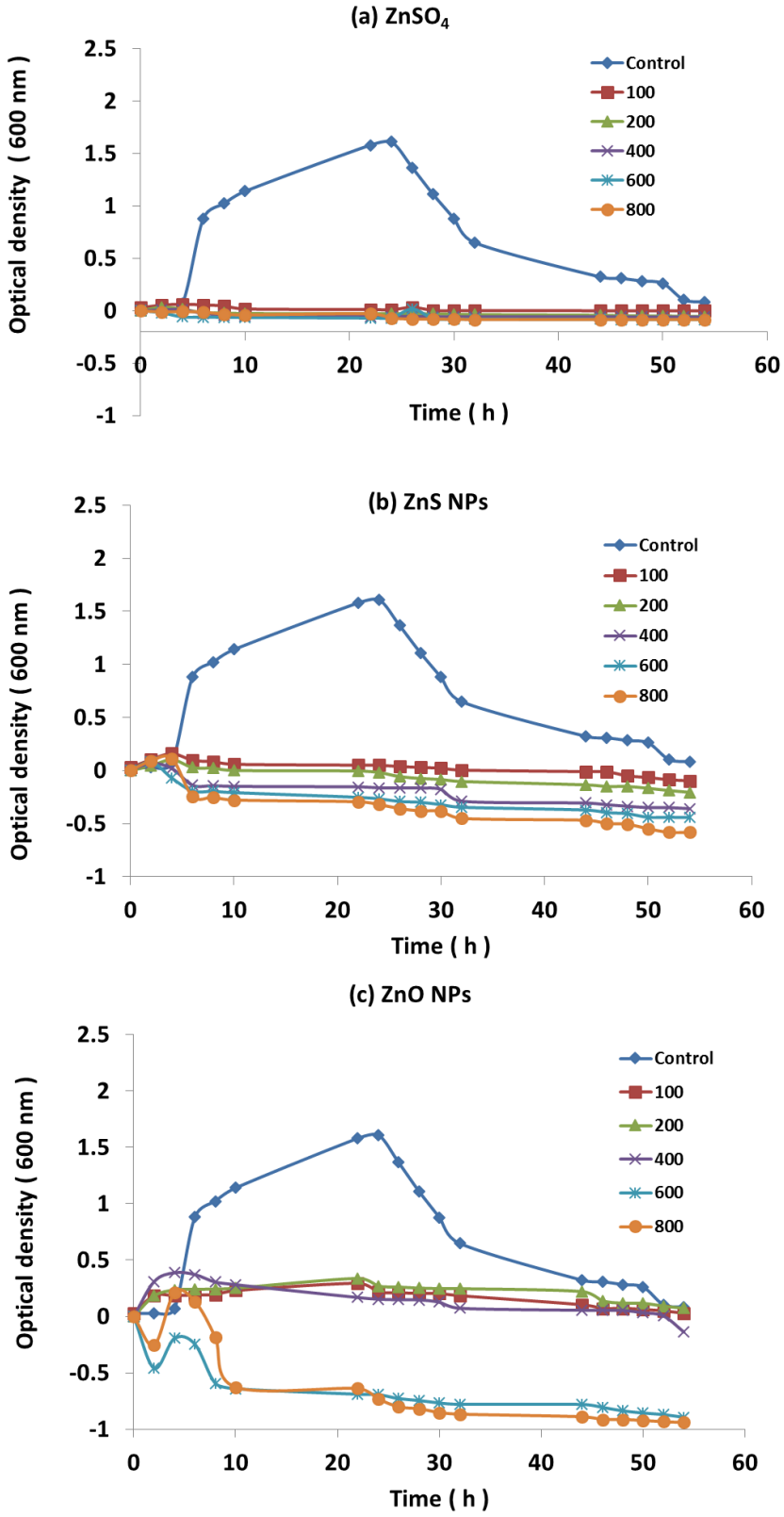


Figure 4.2: Growth of *Pseudomonas brassicacearum* in uncontaminated Hoagland solution and Hoagland solution amended with 100 – 800 mg L^{-1} (a) $ZnSO_4$ (b) ZnS nanoparticles (c) ZnO nanoparticles. Since nanoparticle suspensions contributed to light scattering (absorption) at 600 nm, the absorbance measured at time zero was subtracted from all measurements for nanoparticle suspensions.

The colony forming unit (CFU) method was used to assess whether the bacteria were viable irrespective of growth. Following incubation for 24 h the number of CFU in agar plates containing the two different media investigated are shown in Table 4.2.

Table 4.2 Colony forming units (CFU mL^{-1}) of *Pseudomonas brassicacearum* growing on nutrient and Hoagland media amended with 100- 800 mg L^{-1} of Zn in three different forms. The values are means of 3 replicates. Different letters represent significant differences following Tukey's test ($p<0.05$).

	Nutrient medium			**Hoagland medium**		
Concentration (mg L^{-1} Zn)	**$ZnSO_4$**	**ZnO NPs**	**ZnS NPs**	**$ZnSO_4$**	**ZnO NPs**	**ZnS NPs**
100	9.3×10^5 **b**	19.0×10^5 **b**	17.0×10^5 **b**	1.8×10^5 **b**	1.3×10^5 **b**	1.7×10^5 **b**
200	8.1×10^5 **b**	19.0×10^5 **b**	17.0×10^5 **b**	1.4×10^5 **b**	1.1×10^5 **b**	1.6×10^5 **b**
400	6.4×10^5 **b**	14.4×10^5 **b**	11.7×10^5 **b**	1.0×10^5 **b**	1.0×10^5 **b**	1.2×10^5 **b**
600	6.0×10^5 **b**	9.0×10^5 **b**	11.5×10^5 **b**	1.0×10^5 **b**	1.0×10^5 **b**	1.2×10^5 **b**
800	5.0×10^5 **b**	3.5×10^5 **b**	10.4×10^5 **b**	1.0×10^5 **b**	1.0×10^5 **b**	1.0×10^5 **b**
Control	21×10^6 **a**			1.4×10^6 **a**		

The greatest numbers of colonies were in the control samples. In both media and all Zn treatments, there is a consistent pattern of decreasing number of CFU as Zn concentration increases. Relative to the control for both media, CFU of varying concentrations of different Zn species were remarkably different in both media. The plate count method revealed that some cells remained viable in both the nutrient broth and Hoagland solution with better viability in the nutrient medium relative to Hoagland solution for all Zn treatments. Consistent with the bacterial growth

(nutrient broth experiment) results, there were more viable cells in Zn NPs suspensions relative to $ZnSO_4$ solutions.

4.4.2 Effect of different Zn species on seed germination and seedling growth

Germination of seed started on day 2 of the experiment. The percentage of germination in the control and different Zn and inoculation treatments at 600 mg L^{-1} Zn is shown in Table 4.3. Seed germination responses differed between the Zn treatments. In the uninoculated treatments, the germination percentage of *Brassica juncea* seed was higher in the control (100%) than in all the Zn treatments, but only in the $ZnSO_4$ treatment was there a significant reduction in seed germination compared to the control (Table 4.3). Upon inoculation with *Pseudomonas brassiccacearum* and *Rhizobium leguminosarum,* seed germination increased for all Zn treatments and was not significantly different from the control, apart from the ZnO NPs treatment inoculated with *Pseudomonas brassicacearum* in which on seed germination was significantly reduced to the control.

Table 4.3: Germination percentages of inoculated and uninoculated seeds of *Brassica juncea* in 600 mg L^{-1} Zn, 14 days after planting. Data shown are the mean of three replicates. * indicate significant differences between the control treatments (inoculated and uninoculated) and Zn species following Tukey's test ($p < 0.05$). B1 repesents *Rhizobium leguminosarum* and B2 represents *Pseudomonas brassicacearum).*

Samples	No. of seeds sown	No. of seeds germinated	% Germination
Control	10	10*	100
$ZnSO_4$	10	5.3*	53.4
ZnS	10	9.3	93
ZnO	10	8.6	86
B1 Control	10	10	100
B1 $ZnSO_4$	10	9	90
B1 ZnS	10	9	90
B1 ZnO	10	9	90
Control B2	10	10*	100
B2 $ZnSO_4$	10	9.3	93
B2 ZnS	10	9.3	93
B2 ZnO	10	7.3*	73

The shoot and root lengths measured in *Brassica juncea* seedlings after 14 days growth are shown in Figure 4.3 (a-b).

The interaction between Zn species and bacteria inoculation factors were significant ($p < 0.05$) for shoot length in the 2-way ANOVA so Tukey HSD tests were conducted to determine where significant differences lay between the combined effect of Zn and inoculation treatments, with significant differences indicated by the different letters in Figure 4.3a. In the absence of bacterial inoculation, shoot length after 6 weeks was significantly higher in the control treatment with no added Zn compared

to the treatments with different species of added Zn. However, bacterial inoculation increased shoot length when Zn contamination is present in the form of $ZnSO_4$ than in Zn NPs treatments.

The interaction between the combined effects of Zn species and bacteria inoculation factors were not significant for root length (Figure 4.3b). However, 1-ANOVA tests followed by Tukey HSD tests showed there are significant differences in root length between the Zn species and bacterial inoculation with significant differences indicated by different letters in Figure 4.3b.

The seedlings of *Brassica juncea* were evaluated for tolerance to Zn in the different Zn treatments 14 days after planting. Tolerance index (TI) values of shoots were higher than the values of roots in the same experiments for all Zn treatments (Figure 4.3 c-d). TI values of shoots ranged from 28 to 67% for the uninoculated and 63 to 77% for the inoculated treatments. The TI values of roots ranged from 23 to 58% for the uninoculated and 41 to 68% for inoculated treatments. In uninoculated experiments higher TI values of roots and shoots occured in the Zn NPs treatments than in the $ZnSO_4$ treatment. However, upon inoculation with *Rhizobium leguminosarum* and *Pseudomonas brassicacearum*, the TI values of shoots and roots in the $ZnSO_4$ treatment increased to higher mean values than in the Zn NPs treatments. TI values of shoots and roots were lower in the inoculation experiment with *Pseudomonas brassicacearum* (B2) for the ZnO NPs compared to the ZnS NPs treatment.

From the study results the rank order of the effect on seed germination, seedling growth and tolerance indices of the different Zn species is $ZnSO_4$ > ZnO NPs > ZnS NPs.

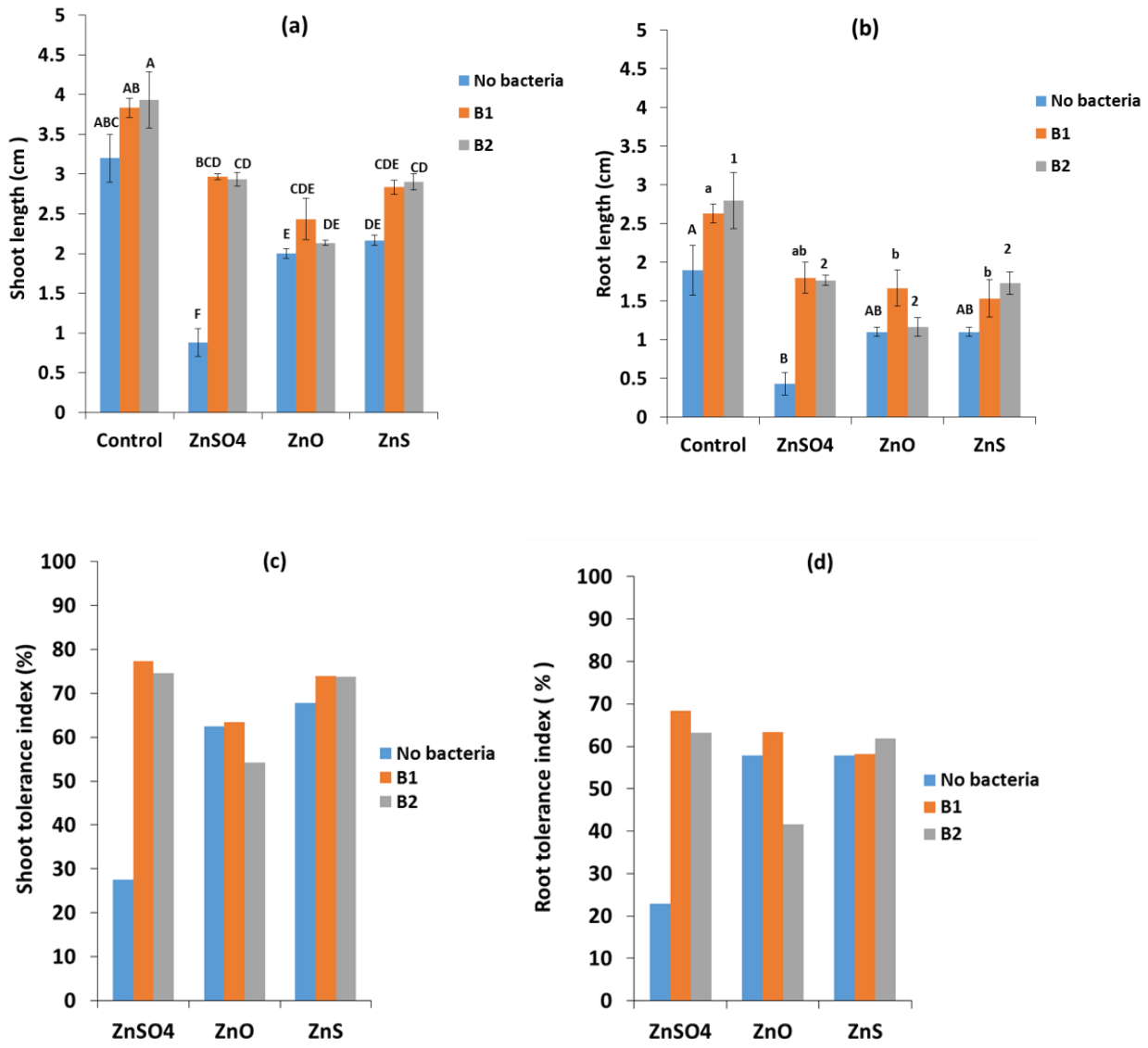


Figure 4.3: Effect of Zn species and bacteria inoculation on growth of *Brassica juncea* seedlings grown in 600 mg L^{-1} Zn, 14 days after planting (a) shoot length, (b) root length, (c) shoot tolerance index, and (d) root tolerance index. B1 repesents *Rhizobium leguminosarum* and B2 represents *Pseudomonas brassicacearum*). Values are means of replicates of 3 petri dishes per treatment and standard mean errors are shown in (a) and (b). Different letters indicate significant differences following Tukey multiple comparison tests ($p<0.05$) between different Zn species treatments in (b) and between the combined effects of Zn species and inoculation treatments in (a).

4.5 Discussion

4.5.1 Zn resistance of *Pseudomonas brassicacearum* and *Rhizobium leguminosarum*

Zn is an essential element for bacterial growth and enzyme activity however, Zn shows toxicity and inhibits microbial processes when in high concentrations (Ali et al., 2012). The result of this study, showed that both bacteria strains investigated (*Rhizobium leguminosarum* and *Pseudomonas brassicacearum*) are resistant to Zn species. Lag time is the initial period in the life of a bacterial growth, when cells are adjusting to new environment before starting exponential growth (Rolfe et al., 2012). The lag time observed for *Pseudomonas brassicacearum* was longer in the Hoagland solution compared to nutrient medium in all Zn treatments. Other studies have reported that heavy metals reduce bacteria growth rate (Mahapatra and Banerjee, 1996). In the bacterial growth experiments, $ZnSO_4$ appeared to be more toxic than ZnO and ZnS nanoparticles, indicating the toxicity of Zn restrained the growth of *Pseudomonas brassicacearum* in $ZnSO_4$ treatment. Bacteria exhibited remarkable resistance to Zn nanoparticles than in $ZnSO_4$ when exposed in nutrient medium. This is probably due to the secretion of exopolysaccharides and entrapment of toxic contaminants (Wilkinson ad Reinhardt, 2005; Chopra, 2007; Miao et al., 2009; Dhas, 2013; Janczarek et al., 2015). The bacteria resistance to Zn nanoparticles may also be dependant on the solubility of the Zn species, suggesting that the small amount of dissolved Zn in ZnO and ZnS nanoparticles treatments induced a comparatively high resistance in bacteria compared to higher dissolved Zn in $ZnSO_4$ treatment.

The plate count method revealed a greater number of bacteria colonies for *Pseudomonas brassicacearum* (Table 4.2) *and Rhizobium leguminosarum* (table not shown) in the nutrient medium than in Hoagland solution (Table 4.2). Spectrophotometer method of optical density (OD) measurement and the plate count method are widely used for determining bacterial populations (Myers et al., 2013; Pan et al., 2014). Result presented in both methods have distinct differences. For example, in the nutrient medium, ZnO nanoparticles treatment had a flat OD at

600 and 800 mg but formed colonies in the plate count method. This is because the spectrophotometric analysis indirectly measures all bacteria (cell biomass), dead and alive, the plate count method reveals information on only live bacteria (Myers et al., 2013; Pan et al., 2014). However, it has been reported that bacteria that are impaired or damaged by environmental conditions are believed to exhibit greater variability in response to added nutrients (Flowers and Ordal, 1979; Roszak and Colwell, 1987). Nutrient medium improved the tolerance of *Pseudomonas brassicacearum* to Zn compared to Hoagland solution. Therefore, selection of appropriate media for metal toxicity study is vital as metal speciation is a function of media components (Shuttleworth and Unz, 1991; Farrell et al., 1993).

Zn could have different species in media that could lead to toxicity in bacteria in this study. Zn complexes with amino acids, polypeptides from yeast extract and peptone in the nutrient broth may decrease the concentration of Zn^{2+}, resulting in lower toxicity in Zn NPs in the nutrient media. Increasing concentration of Zn^{2+} in $ZnSO_4$ treatment may result in higher toxicity to *Pseudomonas brassicacearum* in the nutrient broth. Thus, $ZnSO_4$ was the most toxic of the Zn species examined to the PGPB studied. There were also minor differences in toxicity between the NPs studied (ZnO and ZnS) as displayed by the OD in this study, which could be attributed to solubility. ZnO nanoparticles are more soluble than ZnS; this could be tested by analysing dissolved Zn in suspensions of nanoparticles.

Overall, this study, showed that both *Pseudomonas brassicacearum and Rhizobium leguminosarum* (data not shown) are resistant to Zn NPs more than $ZnSO_4$ at the concentrations to be used in the pot experiment, although growth is compromised at higher concentrations. This implies that they can be used reliably in growth experiments, especially where metal adsorption to soil might provide further protection. However, the bacteria might not be very effective in enhancing the phytoextraction of Zn by plants grown in Hoagland solution in hydroponic conditions.

4.5.2 Phytotoxicity of Zn to seed germination and early seedling growth

Seed germination and seedling growth are relevant phytotoxicity parameters widely used to test the toxicity of chemical species following contaminant release in the environment (USEPA, 1996). These parameters were quantified in this study following exposure of *Brassica juncea* to different Zn species at 600 mg L^{-1} Zn in a simple, rapid and sensitive germination test conducted in petri dishes. The results of study showed that ZnO and ZnS nanoparticles did not significantly affect uninoculated seed germination (Table 4.3). These are consistent with previous studies, which also showed that seed germination is insensitive to nanoparticles exposure (Zhan et al, 2015; Yang et al., 2015). The insensitivity of seeds to Zn nanoparticles exposure in these experiments may be as a result of slow dissolution of Zn^{2+} from ZnO and ZnS NPs, insufficient Zn concentration (dissolved Zn) or selective permeability of the seed coat (Wierzbicka and Obidzinka, 1998; Lin and Xing, 2007; Ma et al., 2010). Although there was no effect on seed germination, the mean shoot and root lengths of seedlings in 600 mg L^{-1} ZnO and ZnS nanoparticles treatments were shorter than the control. Possibly because roots are in direct contact with Zn NPs, thus, the presence of Zn NPs itself or dissoluted Zn^{2+} may interfere with root physiology hence variation in length (Lin and Xing, 2007; Zafar et al., 2016). Metal NPs, including ZnO nanoparticles, have been shown in other studies to adversely affect seedling length (Lin and Xing, 2007; 2008; Zafar et al., 2016). Lin and Xing, (2007) reported that 2000 mg L^{-1} ZnO NPs significantly inhibited root length and terminated root development of corn, radish, ryegrass, cucumber, rape and lettuce. In this study, the phytotoxicity of Zn nanoparticles was minimal as compared with soluble Zn ($ZnSO_4$) in the uninoculated experiments. Exposure to 600 mg L^{-1} of $ZnSO_4$ significantly inhibited seed germination (46.6%) and further significant reduction of shoot and root length compared to the uninoculated control was observed. Previous studies have suggested that the toxicity of metals and metal nanoparticles arises from dissolution and subsequent release of toxic metal ions (Lin and Xing, 2007; Franklin et al., 2007; Ma et al., 2013; Zhang et al., 2015) which is also dependent on their chemical composition, morphology, size and surface characteristics (e.g. charge, coating) (Xu et al., 2010). Zinc is an essential element for plant growth and development; however, when present at elevated concentration,

such as 600 mg L^{-1} Zn in this study will inhibit seed germination, plant growth and development (Alloway, 2008; Broadley et al., 2007). The study findings showed that uninoculated root length was lower in all Zn treatments than shoot length. This is because the radicles (embryonic root of the plant) are the first main tissue to be in direct contact with the Zn treatment thus suggesting that root elongation was more sensitive to all Zn treatments than shoot length (Lin and Xing, 2008; Yang et al., 2015).

The results of the study showed promising effects of inoculation with *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* on seed germination and seedling growth. *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* are known to alleviate unfavourable Zn effects on germination rate, seedling emergence and plant growth in Zn contaminated soil (Adediran et al., 2015; 2016). The present study confirms the beneficial effects of *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* on *Brassica juncea.* Although no significant differences in seed germination between inoculated Zn treatments and the control were observed, inoculation with *Pseudomonas brassicacearum significantly* affected seed germination in ZnO NPs treatments (Table 4.2). It appeared that *Pseudomonas brassicacearum* was less effective in ZnO NPs treatment as germination rate was (26%) inhibited. However, inoculation with *Rhizobium leguminosarum* increased shoot and root length compared to uninoculated ZnO NPs treatment. The seedling growth inhibition observed in uninoculated $ZnSO_4$ treatment was significantly ameliorated upon inoculation with PGPB compared to the Zn nanoparticles treatments.

Possible explanations of the positive effect of PGPB on seedling growth suggested by previous studies include: (a) increased nutrient availability through the solubilisation of unavailable minerals; (b) the production of 1-aminocyclopropane-1-caroxylate (ACC) deaminase by PGPB, thus reducing concentrations of ethylene in the roots of developing plants, thereby increasing root and shoot growth; (d) the production of phytohormones, (d) nitrogen fixation (Bashan and Holguin, 1997; Glick et al., 1995; 2004). Noel et al. (1996) reported significant enhancement of

early seedling root growth of non-leguminous plants (*Brassica species* and lettuce) upon inoculation with *Rhizobium leguminosarum* which was attributed to phytohormone production. However, these PGPB properties were not investigated in the strains in our study.

Overall the PGPB strains used in this study conferred high Zn tolerance on *Brassica juncea*, not only in increasing seed germination and seedling growth but in protecting young seedlings against Zn toxicity compared with the uninoculated plants. Therefore, these plant growth-promoting bacteria are valuable for enhancing the efficiency of phytoextraction of Zn.

4.6 Conclusion

ZnO and ZnS nanoparticles are emerging Zn contaminants owing to their wide application usage in consumer products. Thus their interaction with plants is inevitable, which will affect their uptake, toxicity and transport. In this preliminary study, the toxicity of different Zn species to *Brassica juncea* was assessed with and without PGPB to inform on concentrations for plant growth experiments in which bacteria are also inoculated**.** The results suggest that *Brassica juncea* was tolerant to 600 mg L^{-1} Zn, although phytotoxicity effects on seed germination and seedling growth of *Brassica juncea* were more evident for $ZnSO_4$ than in ZnO and ZnS nanoparticle treatments. Upon bacterial inoculation, Zn toxicity effects on seed germination and seedling growth were diminished in $ZnSO_4$ treatment. Thus both the study plant growth promoting bacteria may play a potential role in alleviating Zn induced toxicity to plants growing in Zn contaminated soil. Although no study has yet examined the role of *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* in remediating soil contaminated with different Zn species, these results offer not only a promising strategy to promote plant growth and ameliorate Zn induced toxicity in hyperaccumulating species but also show that the combination of *Brassica juncea* and the PGPB has potential to be efficient for the phytoextraction of Zn contaminated soil. *Brassica juncea* growing in Zn contaminated soil may have a different tolerance compared to the results from these experiments in the absence of soil since soil is a heterogeneous complex environment and, some of the added Zn will not be bioavailable in soil due to other physico-chemical processes (such as, adsorption, cation exchange, solubility constraints). However, it would be interesting to investigate if the seed germination and seedling growth of *Brassica juncea* exposed to different Zn species and PGPB shows the same trend under greenhouse conditions as in this study. Hence experiments were conducted under greenhouse conditions to investigate in *Brassica juncea* germination and early seedling growth, which are major steps in the plant life cycle.

Chapter 5

5 Probing speciation dependent uptake of trace metals from contaminated soil by metal hyperaccumulating plants

5.1 Introduction

The environment is increasingly being contaminated with toxic metals resulting from natural and anthropogenic activities (Khan et al., 2004; Hou and Al-Tabbaa, 2014). Amongst different potential contaminants, heavy metal contamination has gained considerable attention owing to its potential threat to human health, environmental persistence of metals and detrimental effects on ecosystems (Liu et al., 2009; Batayneh, 2012). Soil contamination with Zn is mostly due to increasing use of Zn in industrial and agricultural activities, such as mining and smelting, dispersal from mine wastes incinerator emissions, excessive applications of Zn-containing fertilisers or pesticides and use of Zn-contaminated sewage sludge (Voegelin et al., 2005; Romeo et al., 2014; Singh et al., 2014). Following deposition, Zn species are generally found in Zn contaminated soil as sphalerite (ZnS) and zincite (ZnO) (Isaure et al., 2002). These two forms of Zn (ZnS and ZnO) are currently amongst the numerous Zn nanomaterials that have recently attracted worldwide attention due to their unique properties for applications in gas sensors, ultraviolet detectors, photovoltaic devices, and personal care products (Rao et al., 2014; Liu et al 2014; Xu et al., 2014). Nanoparticles are seen as materials which have at least one dimension in the size range 1-100 nm (Arruda et al., 2015). Owing to increased nanotechnology applications, nanoparticles may be released into the environment during production, transport and use resulting in adverse effects on the environment such as inhibiting plant growth, and disrupting cell function in plants (Chang et al., 2012; Peralta-Videa et al., 2011; Li et al., 2015; Zhang et al., 2015).

Although Zn is an essential element for life, as it influences plant metabolism and growth process, like other trace metals excessive Zn content in soils may be harmful resulting in detrimental consequences for plant physiology, development and other important metabolic processes (Nagajyoti et al., 2010; Qiao et al., 2014). Thus, there is a need to implement appropriate remedial measures for Zn contaminated

environments. To date, various methods have been proposed for toxic metal removal from the environment. Conventional methods such as landfilling, chemical oxidation, vitrification, and stabilization are disruptive, costly and labour intensive (Lopez et al., 2007). Phytoextraction is a type of phytoremediation that is increasingly being used as an environmentally sustainable remediation technique which involves the use of plants to extract toxic metals from soil into harvestable biomass (Reichman, 2007). A number of plant species, among the *Brassica* genus have been successfully used for the phytoextraction of metals from polluted soil (Su and Wong, 2004). These species have a high capability for accumulating, translocating and tolerating high levels of Zn within plant tissues and are termed hyperaccumulators (Kumar et al., 1995; Lombi et al., 2001; Novo et al., 2013). *Brassica juncea* is currently used to extract and accumulate toxic metals from contaminated soils (Prassad et al., 2003; Bluskov et al., 2005; Meyers et al., 2008; Purakayastha et al., 2008; Dede et al., 2012). Uptake of metals by the roots, transport of metal from roots to shoot, complexation with chelating molecules and compartmentalisation into the vacuole are believed to be the major mechanisms by which hyperaccumulators alleviate plant stress from toxic metals (Hall, 2002; McGrath and Zhao, 2003; Lu et al., 2013).

In metal contaminated soil, the fraction of the total metal concentration which can be readily mobilised in the soil environment and taken up by plant roots is considered as the bioavailable fraction (Vamerali et al., 2010; Bhargava et al., 2012). Thus, current literature suggests that consideration of metal bioavailability in soils is essential decisive for the success of phytoextraction (Li and Ramakrishna, 2011). The biogeochemical Zn behaviour and its potential effects on plants are strongly influenced by its speciation. Speciation is the existence or the distribution of a metal in different chemical forms in a system (Shahid et al., 2011; Cough et al., 2012). Therefore, the degrees of Zn toxicity, bioavailability and mobility in soils are controlled by Zn speciation (Voegelin et al., 2005).

More information about nanoparticles including their accumulation, transport and toxicity is needed to understand their behaviour in the environment (Ma et al.,

2013; Nair et al., 2010; Zhang et al., 2015). The aims of this study are (i) to compare the effect of different Zn species on plant growth; (ii) to compare the uptake and toxicity of Zn when the Zn is present in the form of soluble $ZnSO_4$ and nanoparticulate ZnS and ZnO on *Brassica juncea* (L.) Czern to inform the associated environmental risks; and (iii) to investigate the fractionation of Zn in soil without the presence of plants.

It was hypothesised that:

(i) Zn speciation will influence plant growth under different zinc contamination.

(ii) There will be differences in uptake and Zn toxicity in *Brassica juncea* based on the form Zn was exposed in soil.

5.2 Materials and Methods

5.2.1 Zinc forms

Zinc sulphate ($ZnSO_4.7H_2O$) and ZnO nanoparticle were purchased from Sigma Aldrich UK and stored according to the vendor's instructions while ZnS nanoparticles were synthesised in the laboratory by using a chemical precipitation method (Ganguly et al., 2014) as decribed in Chapter 3.

The morphology of ZnS nanoparticles was examined using X-ray diffraction (XRD) in order to identify their crystal phase and microstructural investigation was carried by transmission electron microscopy (TEM) (Philips CM 120) as described in Chapter 3.

5.2.2 Soil preparation and treatment

A natural topsoil without peat amendment was used in this study. This is due to the fact that high organic matter content in compost will modify metal mobility and availability (Al Chami et al., 2013). Topsoil encourages rooting, airflow and drainage and also a representative of field condition. Westland topsoil was purchased from Dobbies Garden Centre Edinburgh UK.

The soil was air dried, crushed and finely sieved through a 2 mm stainless steel sieve to disaggregate clumps and remove any coarse debris present. The prepared soil was mixed with 10% sand by volume to aid drainage. Selected properties of the soil determined using the methods described in Chapter 3 are listed in Table 5.1.

Table 5.1: The physical and chemical properties of experimental soil (Values are means of the analysis of three sub-samples).

Parameters	Value
Moisture content (%)	26.2
Organic matter content (% LOI)	15.4
pH	6.2
N (mg g^{-1})	1.79
P (mg g^{-1})	0.30
K (mg g^{-1})	8.49
Zn (mg g^{-1})	0.03
Fe (mg g^{-1})	13.6
Ca (mg / kg)	2078.5
Mg (mg / kg)	279.2
Mn (mg / kg)	20.6
Na (mg / kg)	43.5

5.2.3 Pot experimental design

A pot experiment was conducted to assess the effect on plant growth when elevated Zn concentrations are added to soil in the form of soluble Zn or as nanoparticulate, ZnS and ZnO. The pot experiment was conducted in a greenhouse set to provide a day/night temperature of 21^{o}C in a 18 h photoperiod at a photosynthetic photon flux density (PPFD) of 150 µmol m $^{-2}$ s^{-1} provided by cool white fluorescent bulbs. The experimental design consisted of each treatment in triplicate pots. The air dried soil was weighed and amended with 600 mg Zn kg^{-1} of $ZnSO_4$, ZnS and ZnO nanoparticles in solid form. The spiked soil sample was mixed by hand for 1 hr to produce a homogenous sample. 1 kg of spiked (Zn, ZnO and ZnS) or un-spiked soil (control) was placed in 2.15 L pots. Both planted and unplanted pots (Table 5.2) and were left to equilibrate for a week in the greenhouse.

Brassica juncea (L.) CZern seeds (purchased from Sow Seeds Ltd., UK) were surface sterilised in 5% NaClO for 15 mins, then rinsed with deionised water thrice. 5 sterilised seeds were directly sown into individual planted pots. Emergent seedlings

were thinned out to 3 plants per pot at 12 days after planting. Planted and unplanted pots were watered with 100 ml tap water twice weekly to maintain soil moisture during plant growth. Leachates from all pots were collected using individual pot saucers and returned back to the soil.

Growth parameters of *Brassica juncea* (L.) CZern were measured at different growing stages starting from seedling. Plant height, from the soil surface to the tip of the longest shoot, was measured once a week on each replicate and for each treatment. Metal-related phytotoxicity in each planted pot was evaluated by monitoring growth rate based on (i) plant height, (ii) leaf count (iii) dry biomass (iv) other observations such as leaf chlorosis, necrosis, drying and senescence.

Soils from the unplanted pots were also evaluated to observe non-plant effects of Zn from 0 to 6 weeks. The planted pots were harvested 6 weeks after planting.

Table 5.2 Description of experimental treatments

Treatment	**Description**
Control	Control without Zn treatment
$ZnSO_4$	*B. juncea* grown on $ZnSO_4$ soil
ZnS	*B. juncea* grown on ZnS nanoparticles soil
ZnO	*B. juncea* grown on ZnO nanoparticles soil
sControl	Unplanted control no Zn treatment
$sZnSO_4$	Unplanted soil treated with $ZnSO_4$
sZnS	Unplanted soil treated with ZnS nanoparticles
sZnO	Unplanted soil treated with ZnO nanoparticles

5.2.4 Plant and soil analysis

Following plant harvest, shoots were cut at their base, and roots were separated. Both plant materials were carefully washed with tap water and rinsed with deionized water until soil particles were completely removed. Samples were oven

dried at 65^{o} C for 72 h and dry weight was recorded. Total Zn content of plant material was determined using a wet acid digestion method (Allen et al., 1974; Lowther, 1980). Ground samples were digested in 2 mL conc. H_2SO_4 and 0.75 mL H_2O_2, in a heating block at 320^{o} C for 6 h. Soil total Zn content was determined using a dry ashing method (Allen et al., 1974) by using 1 mL HNO_3 + 6 mL HCl. Digests were filtered with Whatman No. 44 filter paper and made up to 50 ml volume with deionised water, stored at 4^{o}C and analysed within a week (described in detail in Chapter 3). Zn concentration in the digests were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES) (PerkinElmer Optima 5300DV). Blanks from both digests were deducted from the analytical result.

5.2.5 Chlorophyll content

After plant harvest 0.5 g of fresh *Brassica juncea* leaves was weighed and torn into pieces. Torn leaves were ground and homogenised in a vortex for 30 s. Chlorophyll content was extracted in 2 ml of 80% acetone. The extract was transferred to centrifuge tubes and stored at 4°C in the dark for 24 h. and then centrifuged at 3000 g for 15 min. Chlorophyll contents was determined spectrophotometrically at 663 and 645 nm. Chlorophyll content was expressed as mg g^{-1} fresh weight and calculated as described in Chapter 3.

5.2.6 Analysis of Zn soil pore water

Soil pore water was extracted by centrifugation (Di Bonito, 2005; Krishnamurti et al., 2013; Ma et al., 2013) of 150 g of Zn spiked soil ($ZnSO_4$, ZnS and ZnO nanoparticles) at the start of experiment and after 6 weeks (detailed description in Chapter 3).

5.3 Statistical analysis

The means and standard error (SE) of all measured parameters were calculated for each treatment. The comparison of plant height, chlorophyll content, roots and shoot biomass, was done by one-way analysis of variance ANOVA using Minitab 17 software (Minitab ™ Inc, USA). All treatment means were normally distributed using the Anderson-Darling' normality test. If a significant difference was observed

between treatments, post- hoc Tukey tests were used for multiple comparisons with significance level of $p < 0.05$.

5.4 Results

5.4.1 Phase characterization of ZnS nanoparticles

The XRD analysis of the synthesised ZnS nanoparticles showed three broad peaks at 2Θ angle of 28.5, 48.2 and 56.50 (Figure 5.1) corresponding to the lattice planes of (111), (220) and (311) of ZnS sphalerite structure. This result suggests that the crystal structure of the sample accords well with that of the ZnS with the standard code (ICSD No. 01-0729269). Furthermore, there are no diffraction peaks from ZnS impurities. The crystallite size was 86.5 Å (8.65 nm) as calculated from the Debye-Scherrer formula (see Chapter 3).

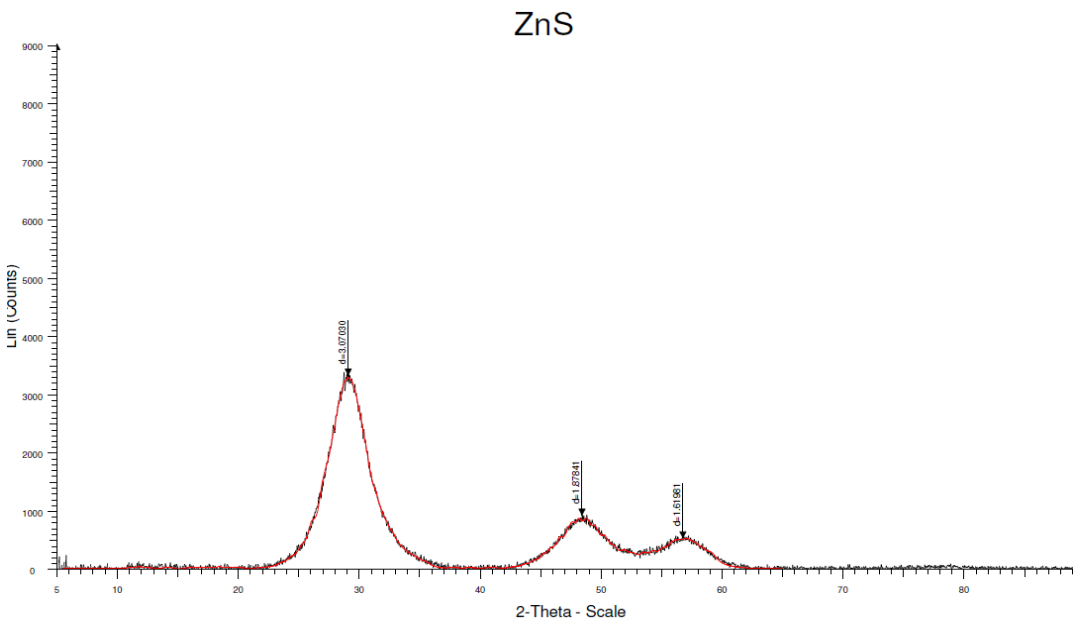


Figure 5.1 The XRD diffractogram pattern of synthesised ZnS nanoparticles

TEM images of the synthesised ZnS nanoparticles (Figure 5.2) indicate that the material occurred in clusters.

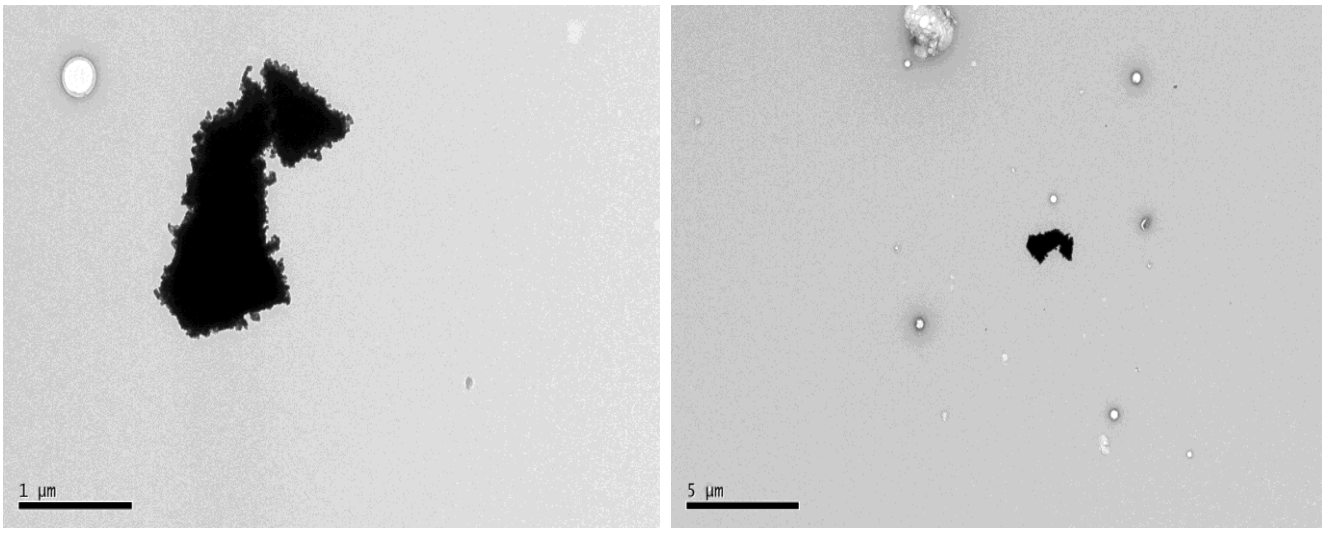


Figure 5.2. Transmission electron micrograph of the synthesized ZnS nanoparticles.

5.4.2 Effect of Zn speciation on growth of *Brassica juncea (*L.) Czern

Plant growth in the pot experiment in the green house was monitored weekly from seed sowing to harvest. There was no measurable plant growth in $ZnSO_4$ treatment 10 days after germination compared to the other Zn treatments and control (Figure 5.3).

Figure 5.3: Variation in plant growth 10 days after sowing. Control without Zn (white), $ZnSO_4$ (red), ZnS nanoparticles (yellow) and ZnO nanoparticles (green).

Figures 5.4 a –d clearly shows the effect of Zn speciation on the growth of *Brassica juncea* (L.) *CZern* plants exposed to 600 mg Zn kg^{-1} of $ZnSO_4$, ZnS and ZnO nanoparticles for 6 weeks. The effect of Zn speciation on plant height 600 mg Zn kg^{-1} showed plants in the control had the greatest height. In comparison to the control, soluble Zn ($ZnSO_4$. $7H_2O$) was the most toxic as it significantly reduced plant height to 5.2 cm (Figure 5.4 a). Furthermore, visible symptoms of toxicity (yellowing of leaves) were observed on the *Brassica juncea* (L.) Czern grown for 6 weeks in soil amended with $ZnSO_4$ (Figure 5.5). These symptoms became more severe with increasing exposure time as the leaves of the plants began to wilt and fall off after 6 weeks of growth. Interestingly, *Brassica juncea* (L.) *CZern* grown in soil exposed to ZnS and ZnO nanoparticles are not significantly different in height. Exposure of *Brassica juncea* to ZnO and ZnS nanoparticles showed better growth and tolerance after 6 weeks of growth compared to plants exposed to $ZnSO_4$ as there were no symptoms of toxicity throughout the 6 weeks growth period.

The number of leaves per plant was counted and the mean number of leaves per pot was calculated weekly for 6 weeks. At the end of week 6 the number of leaves showed the same pattern between treatments as the plant height results, but the differences between the control and different Zn species were not significant for (Figure 5.4 b).

The root and shoot biomass of *Brassica juncea (L.) CZern* after 6 weeks of growth (Figures 5.4 c) showed similar patterns by Zn treatment as the plant height results. Shoot and root biomass were both significantly lower for all Zn treatments compared to the controls.

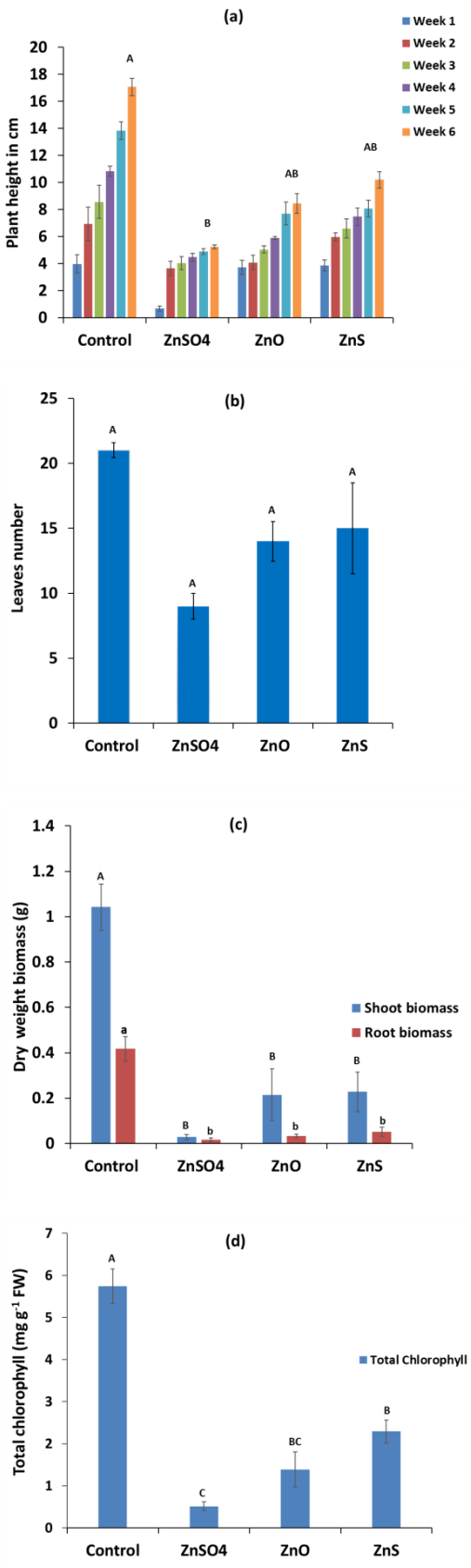


Figure 5.4: *Brassica juncea* grown in 600 mg Zn kg^{-1} treatments. (a) plant height, (b) number of leaves, (c) shoot and root biomass and (d) total chlorophyll 6 weeks

after planting. Values are means of three replicates and bars are the standard mean error of replicates. Different letters indicate significant differences $p < 0.05$.

Chlorophyll content in leaves of *Brassica juncea (L.) Czern* was significantly lower in all Zn treatments after six weeks of growth compared to the planted control pots with no added Zn (Figure 5.4 d). Leaves from the ZnS nanoparticle treatment had a significantly higher chlorophyll content compared to the $ZnSO_4$.

Overall the results show that there were systematic Zn speciation-dependent trends in plant growth between the three Zn treatments. At the Zn concentration tested, $ZnSO_4$ was more toxic than the nanoparticle treatments as plants were free of symptoms of toxicity after 6 weeks of growth. All parameters for monitoring toxicity showed a consistent trend, with toxicity in the order: $ZnSO_4$ > ZnO > ZnS nanoparticles.

Figure 5.5: Typical chlorosis of leaves of *Brassica juncea (L.) Czern* after 6 weeks of plant growth caused by 600 mg/kg $ZnSO_4$ treatment.

5.4.3 Zn concentration in plant material

Zinc concentrations in dry biomass of *Brassica juncea* at harvest after 6 weeks are shown in Figure 5.6. As expected, Zn concentrations in both shoot and root biomass were high in the added Zn treatments compared to the uncontaminated controls. Shoot Zn differed between the Zn treatments at 600 mg Zn kg^{-1}. The highest shoot

Zn concentration occurred in the $ZnSO_4$ treatment and was higher than the other Zn nanoparticle treatments.

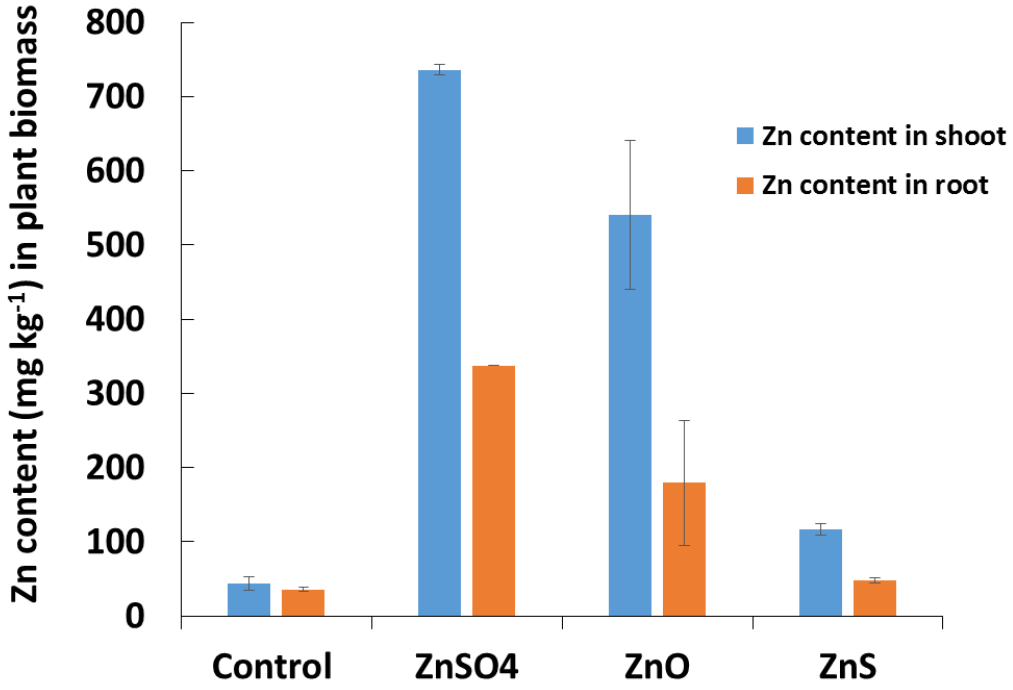


Figure 5.6: Zn concentrations in shoots and roots 6 weeks after planting in soils with different Zn contamination characteristics soil with 600 mg kg^{-1} Zn added. Bars are the standard error of the mean of two mixed samples per treatment.

Root Zn concentrations (Figure 5.6) showed a similar pattern to shoot Zn concentrations. Zn concentration was more in the roots of *Brassica juncea* treated with $ZnSO_4$ compared to the ZnO and ZnS nanoparticles treatments.

5.4.4 Zn concentrations in soil pore water

Zn concentrations in soil pore water at the start and end of the experiment from the unplanted control and different Zn treatments applied at 600 mg Zn kg^{-1} are shown in Figure 5.7. Soil pore water data showed time dependent changes of different Zn treatments in soil pore water Zn concentrations from 0 to 6 weeks.

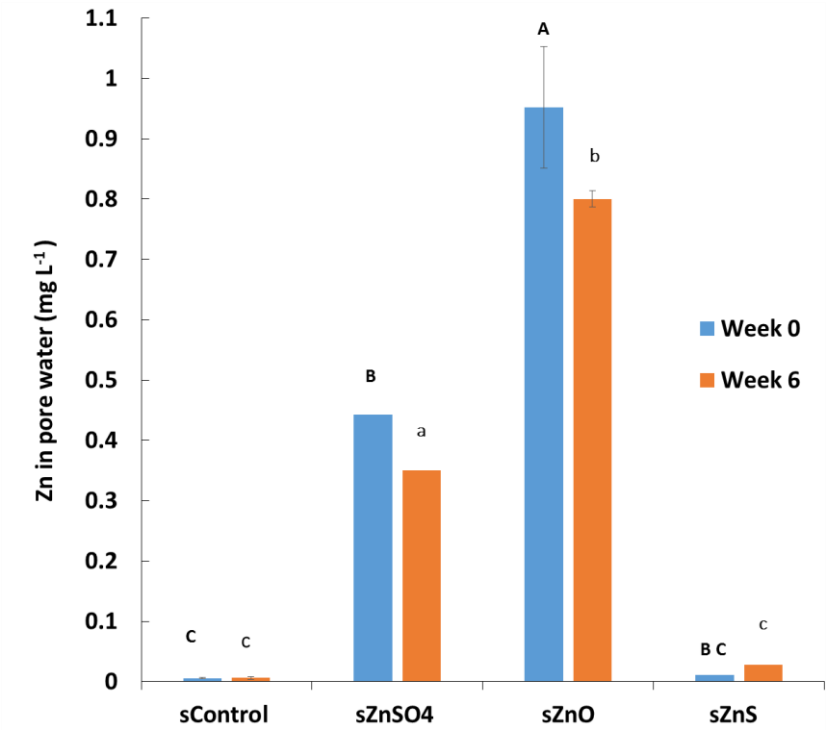


Figure 5.7: Zn concentration in soil pore water from unplanted pots with different Zn treatments extracted at week 0 and 6 weeks in soil. Data are means and standard error of two pots. Different letters indicate significant differences (P< 0.05).

Soil pore water from the uncontaminated control had a significantly lower Zn concentration than the $ZnSO_4$ and ZnO treatments at the start and end of the experiment. At week 0, Zn concentrations in the soil pore water were significantly different between $ZnSO_4$ and ZnO treatments. At week 6, Zn concentrations were significantly different for all Zn treatments. By week 6, soil pore water Zn concentrations had decreased in the ZnO nanoparticle and $ZnSO_4$ treatments but not in the ZnS nanoparticle treatments. Overall, soil Zn was mobilized to pore water in the following order: ZnO nanoparticles > $ZnSO_4$ > ZnS nanoparticles.

5.5 Discussion

It was hypothesized that Zn speciation influences plant growth in Zn contaminated soil. The effect of three different forms of Zn was investigated on plant growth, Zn accumulation and toxicity on a hyperaccumulator species (*Brassica juncea* (L.) Czern), known for its tolerance, capacity to extract and accumulate Zn from the environment (Purakayastha et al., 2008; Dede et al., 2012). Results of the effects of different Zn forms ($ZnSO_4$, ZnS and ZnO nanoparticles) on the growth of *Brassica juncea* (L.) Czern in Zn contaminated soil are discussed below.

5.5.1 Zn speciation effects on plant growth

Zinc is a micronutrient required in plant health, playing an important role in plant metabolism by influencing the activities of hydrogenase and carbonic anhydrase, as well as in the synthesis of tryptophan, a precursor to indoleacetic acid synthesis (Hafeez et al., 2013). The effects of the different Zn species on *Brassica juncea (L.) Czern* grown in soil contaminated with 600 mg Zn kg^{-1} were assessed using four variables - plant height, number of leaves, biomass and chlorophyll content and other visual observations. Systematic speciation-dependent trends in plant growth for all three Zn treatments were observed in this study. In the $ZnSO_4$ treatment plant height, number of leaves and plant biomass were lower compared with the control and Zn nanoparticles treatments (ZnO and ZnS). It is well established that Zn stimulates *Brassica juncea* growth at low concentration (Grewal and Graham, 1997; Singh and Sinha 2004; Prassad et al., 1999) and at higher concentration causes significant suppression in plant growth. After 6 weeks of plant growth at 600 mg Zn kg^{-1} , chlorophyll content was significantly reduced for all Zn treatments than in the control, with the most reduced chlorophyll content in $ZnSO_4$ (Figure 5.4 d). A likely cause of this reduction is the replacement by dissolved Zn^{2+} in Zn species of the central Mg^{2+} of chlorophyll, resulting in interference with synthesis of photosynthetic pigment (Kupper and Anderson, 2016). Novo et al. (2014) reported 24.4 % and 35% in chlorophyll reduction in *Brassica juncea* exposed to high concentrations of 900 and 1800 mg Zn kg^{-1}. It was also observed that excess ZnO

concentration (1000 mg L^{-1}) significantly decreased total chlorophyll in *Brassica juncea* (Roa and Shehawat, 2014).

Our data suggest that *Brassica juncea* exposure to ZnO and ZnS nanoparticles had less impact on all plant growth parameters compared with $ZnSO_4$. Different effects of ZnO nanoparticles on plant growth and biomass have been reported. Priester et al. (2012) and Lin and Xing (2008) reported the reduction in root length and biomass in soil treated with ZnO nanoparticles in edible plants. Wang et al., (2013) suggested that $ZnCl_2$ was more toxic than ZnO nanoparticles in plants exposed up to 500 mg kg^{-1} ZnO nanoparticles. In addition, Prassad et al., (2012) concurred with the results of the present study that at 1000 ppm of ZnO nanoparticles, ZnO nanoparticle had less impact on seed germination, biomass and vigour than groundnut seed treated with 1000 ppm $ZnSO_4$. In this study, there were no visual symptoms of toxicity elicited by ZnO and ZnS nanoparticles in *Brassica juncea*. *Brassica juncea* survived over the 6 week growing period in ZnO and ZnS nanoparticle treatments compared to in the $ZnSO_4$ treated soil.

$ZnSO_4$ is highly soluble and, as such toxicity was higher, affecting plant growth more than Zn applied as ZnS and ZnO nanoparticles. This corroborates that at the concentration applied, soluble zinc ($ZnSO_4$) elicited wilting, necrosis, chlorosis, and biomass decline in *Brassica juncea* which is typical of Zn toxicity (Bonnet et al., 2000; Kopponen et al., 2001; Khudsar et al., 2004; Jain et al., 2010). Metal bioavailability is critically dependent on bioaccessibility and, since soluble and uncomplexed Zn is more bioaccessible (Arnold et al., 2007), the observed toxicity trends are entirely consistent with respect to $ZnSO_4$ and the nanoparticulate forms. Whilst this result is interpreted as the direct response of *Brassica juncea* to Zn, there may have been other unforeseen or indirect effects which were not determined in this study. For instance, the concentration of Zn (600 mg Zn kg^{-1}) added to the study soil, may have altered the bioavailability of other essential elements. Complex interactions of Zn with Ca, Mg, Fe, P, may affect the availability of each other in soils and their status in plants through the processes of growth, absorption, distribution or utilization (Loneragan and Webb, 1993; Siedlecka, 2014), resulting in Zn toxicity. Shetty et al.

(1994) reported that growth inhibition was due to interference of Zn with P uptake by plants. Davies and Parker, (1993) reported that Zn toxicity was correlated with Ca: Zn ratio and reduced stem biomass. Chavan and Banerjee (1980) reported that Zn toxicity in plants appears to be due to Fe deficiency. Another factor is the soil pH which influences not only metal bioavailability but also controls metal uptake into plant root. The soil pH used in this study as seen in Table 5.1 was 6.2. This pH falls within the range for optimum growth of *Brassica juncea* (McCaffery, 2009). However, due to the acidic nature of the study soil, the greater Zn bioavailability may enhance Zn uptake (Pilon-Smits, 2005; Rout and Das, 2009), thus inducing phytotoxicity in *Brassica juncea* growth and biomass. Studies have reported that the toxic effects of Zn on *Brassicaceae* species (*Brassica juncea*, *Thlaspi caerulescens*) was mainly due to presence of Zn (Ebbs and Kochian, 1997; Schwartz et al., 1999; Ebbs and Uchil, 2008).

This is the first study to the best of our knowledge, to investigate the plant response to ZnS nanoparticle-contaminated soil as there is no information on their fate and toxicity to plants. This study suggests that ZnO and ZnS nanoparticles are less toxic than $ZnSO_4$ in terms of plant growth, development and yield. However, the reason for the lesser impacts of ZnS and ZnO nanoparticles on plant growth compared with $ZnSO_4$ growth might be the adherence of nanoparticles to soil particles. Nanoparticles have the potential to aggregate (Figure 5.2) which may increase their size and reduce their mobility and toxicity (Martinez-Fernandez et al., 2015). It has been reported that the effects of nanoparticles on plant growth is solely dependent on the type of nanoparticles, size, concentration, plant species and plant substrate (culture medium, hydroponics and soil) (Ma et al., 2010; Aruda et al., 2015).

5.5.2 Uptake of Zn by *Brassica juncea* (L.) Czern.

Uptake of Zn by *Brassica juncea* grown in soils with added $ZnSO_4$, ZnS and ZnO nanoparticles was studied. The concentrations of Zn in roots and shoot of *Brassica juncea* varied across different treatments (Figure 5.6). The maximum Zn

concentration was observed more in shoots than in roots of *Brassica juncea* grown in $ZnSO_4$ contaminated soil, indicating high mobility of Zn from soil to root. Similarly, Zn concentration was higher in shoots of *Brassica juncea* than in roots in soil amended with ZnO or ZnS nanoparticles. This may suggest that ZnS and ZnO nanoparticles underwent slow dissolution within the soil, hence assisting translocation into shoots. The uptake of nanoparticles in plant parts is not only related to surface properties, size, shape, particle composition, and growth matrix, amongst others but also dependent on the plant species (Siddiqui et al., 2015). Measuring Zn concentration in plant tissues alone is not sufficient to evaluate the ability of plant to extract Zn from soil because it does not consider plant biomass. Shoot biomass and metal concentration will be the most important indicators for plant Zn removal from Zn contaminated soil and which influence phytoextraction efficiency (Lofty et al., 2012; Bauddh and Singh, 2012), bioaccumulation and translocation factors (Bhuiyan et al., 2016). These contributing factors (bioaccumulation and translocation factors) will be evaluated in the next chapter.

5.5.3 Effects of Zn speciation in soil pore water

The pore water metal content is the readily available fraction of the total metal content in soil (Krishnamurti et al., 2015). The pore water Zn concentrations indicated that Zn release in the ZnO nanoparticles treated soil was greater than the soils with added $ZnSO_4$ and ZnS nanoparticles (Figure 5.7). Soil pH is an important parameter likely to be involved in the bioavailability of Zn (Waalewinjin et al., 2013a, b; Dimkpa et al., 2014). Although the pH of the Zn soil pore water was not ascertained in this study, taking the study soil pH into account, acidic conditions of the study soil would favour dissolution for ZnO nanoparticles (Yamabi and Imai, 2002; Mahdavi et al., 2012). Several studies have been reported that dissolution of Zn oxide nanoparticles in soils from within 1 day (Scheckel et al., 2010) to within a few months to years of soil incubation (Voegelin et al., 2005; Brennan and Bolland, 2006). Higher dissolved Zn concentrations in pore water in $ZnSO_4$ spiked soil is also expected to be related to the higher solubility in water of Zn salts than ZnS nanoparticles. The lowest Zn concentration in soil pore water occurred in the ZnS nanoparticles-treated soil probably due to slow dissolution of ZnS nanoparticles. Zn

concentrations in soil pore water in the unplanted ZnS nanoparticles treatment increased slightly from week 0 to week 6 (Figure 5.7). This suggests that dissolution of ZnS nanoparticles in soil can be expected to occur on the time scales of years (Voegelin et al., 2011). Agglomeration of ZnS nanoparticles and precipitation as carbonates and phosphates (Ress et al., 2015) might also be a factor hindering Zn release in soil, explaining why ZnS was less mobile than $ZnSO_4$ in the experimental system, favouring plant growth and decreased metal uptake in *Brassica juncea*. While metal dissolved in the soil solution constitutes the most readily phytoavailable pool of metals in soil, a significant proportion of the metals in the soil solution in most soils is not in a form which can be absorbed by plant roots (Hamon and McLaughlin, 2003). Based on the higher Zn concentration measured in plant tissue (Figure 5.6), soluble Zn ($ZnSO_4$) was more phytotoxic than ZnO and ZnS nanoparticles, corresponding with the higher solubility of the Zn salt. However, in soil pore water, higher Zn concentrations occurred in the ZnO nanoparticles treatment. Thus, the concentration of Zn in Zn soil pore water does not explain the patterns of Zn uptake in plant in this study. The higher soil pore water Zn concentrations in the ZnO nanoparticles treatment compared to the soluble Zn ($ZnSO_4$) treatment suggests, that ZnO nanoparticles may also be toxic to plants by releasing Zn^{2+} ions which may be detrimental to plants. However, there are no conclusive data in this study that indicates ZnO toxicity effects on plants as compared with $ZnSO_4$ in Zn contaminated soil.

5.6 Conclusion

Knowledge of Zn speciation is important in understanding its bioavailability and toxicity in Zn contaminated soil. The results presented here show that, despite the release of Zn ions, ZnO and ZnS nanoparticles did not produce visible signs of Zn toxicity including necrosis, stunting, and chlorosis or wilting in *Brassica juncea*. The nanoparticles effect on plant growth was lower than in soils treated with soluble Zn ($ZnSO_4$). Thus, this study suggests that the toxicity of $ZnSO_4$ to plants is due to Zn speciation problem.

The uptake and accumulation of Zn in plant tissue are vital in evaluating the role of plant in extraction or removal of Zn from contaminated soil. The key findings findings from this research support the fact that release of Zn from $ZnSO_4$, ZnS and ZnO nanoparticles influences Zn uptake, accumulation and toxicity in plants. The results of the present study revealed that *Brassica j*uncea accumulated considerable biomass and accumulated Zn during the experimental period. *Brassica juncea* may therefore be considered as an effective hyperaccumulator for remediation of soils contaminated with Zn. The next stage of the research aimed to assess whether metal resistant plant growth promoting bacteria increased the efficiency of phytoextraction by *Brassica juncea* of Zn in soil contaminated with Zn.

Chapter 6

6 Mechanisms behind the plant growth-promoting effect of bacteria in soils contaminated with different species of Zn

6.1 Introduction

Accumulation of inorganic contaminants has occurred extensively in soil ecosystems (Kim et al., 2015). Plants which are the part of the ecosystem will inevitably interact with metals thus influencing their uptake and accumulation in plant biomass and in turn affect their spatial distribution and speciation (Lin and Xing, 2007; Lin et al., 2009; Ma et al., 2010). Although Zn is specifically vital for healthy plant life, previous studies have reported that excess Zn is detrimental to plants due to its high persistency in soil, thus can induce physiological, morphological and biochemical dysfunctions in plants such as reduced plant growth, chlorophyll production, seed reduction chlorosis and necrosis (Reichman, 2002; Rascio and Navari-Izzo, 2011). The effects of Zn speciation on the growth of *Brassica juncea* was demonstrated in Chapter 5 to show that plant growth in Zn contaminated soil was dependent on the form in which Zn was exposed to soil (Chapter 5). This effect was linked to Zn solubility and mobility in soil.

Hyperaccumulating plants such as *Brassica juncea* are excellent species in extracting Zn in soil (Salt et al., 1998; Adediran et al., 2015). However, the success of this environmentally sustainable technology may not only depend on the plant itself but most effectively by utilizing plant growth promoting bacteria (PGPBs). These PGPBs are free living bacteria and can also form symbiotic relationships with plants resulting in promoting plant growth (Glick, 2012), such interaction between these PGPB and metals including nanoparticles could affect plant responses. Several studies have demonstrated that inoculation of plants with metal-resistant PGPB is efficient in ameliorating plant stress in metal contaminated soil (Weyen et al., 2009; Ma et al., 2013; Phieler et al., 2015; Adediran et al., 2015; 2016), and it was shown in Chapter 4 that the presence of bacteria improved germination and seedling growth. Plant growth promoting bacteria can alter metal speciation, increase metal

solubility and affect plant growth by secretion of phytohormones (Zhuang et al., 2007), production of chelators and siderophores (Dimkpa et al., 2009), acidification and biomineralization (Abou-Shanab et al., 2008).

To date, very little is known about the mechanism of the uptake and accumulation of nanoparticles in plants, including their transport to their biological parts (Ju-Nam and Lead, 2008). Most studies of nanoparticles uptake have been conducted on edible plants (Nair et al., 2010; Rico et al., 2011). Lin and Xing (2008) have reported the accumulation and distribution of ZnO nanoparticles in ryegrass roots by transmission electron microscopy. On the contrary, Lopez-Moreno et al. (2010) reported the absence of ZnO nanoparticles in soybean roots even at high concentration (4000 mg L^{-1}) by X-ray absorption spectroscopy. Recently, Hernadez-Viezcas et al. (2013) reported that Zn accumulated in the form of Zn citrate in soybeans (Glycine max) under ZnO nanoparticles treatment using synchrotron-based XAS and μ-XRF analysis. However, there was no basal comparison between soluble Zn and nanomaterial in these studies. Moreover, none of these studies were targeted for phytoremediation assisted by PGPBs. However, using synchrotron based XAS and μ-XRF on Zn speciation study targeted at Zn phytoextraction, Adediran et al. (2015) reported that Zn was in form of sulfate in uninoculated roots of *Brassica juncea* while in roots inoculated with PGPB, Zn was in form of phytate and cysteine under treatment with 400-600 mg Zn Kg^{-1} of $ZnSO_4$. Thus, a key focus of this study was to compare Zn responses of inoculated and uninoculated *Brassica juncea (L.) Czern* exposed to soluble zinc ($ZnSO_4$) and Zn nanoparticles (ZnO and ZnS) in a natural soil devoid of peat amendment. This is to avoid added sulphate being reduced by organic matter acting as an electron donor to sulphate reducing bacteria if the soil becomes anoxic. The information on the speciation of Zn following the addition of soluble Zn and nanoparticulate Zn to soil and in association with PGPBs is of importance for a realistic assessment of the potential effectiveness of phytoremediation. The aims of this study were (i) to assess the role of plant growth promoting bacteria (PGPB) on growth of *Brassica juncea* exposed to different forms of Zinc species (ii) to compare the uptake, accumulation and translocation between inoculated and uninoculated plants, (iii) to evaluate Zn distribution in inoculated

and uninoculated roots of *Brassica juncea* in response to exposure to different Zn forms and, (iv) to examine the mechanism of Zn tolerance and hyperaccumulation in *Brassica juncea*.

6.2 Material and methods

6.2.1 Zinc forms

Zn in the form of $ZnSO_4$, ZnS and ZnO nanoparticles was used in this study. Zinc sulphate ($ZnSO_4.7H_2O$) and ZnO nanoparticles were purchased from Sigma Aldrich UK (35 nm average nanoparticle size) and stored according to the vendor's instructions while ZnS nanoparticles were synthesised in our laboratory using a chemical precipitation method (Ganguly et al., 2014) (see Chapter 3 for detailed ZnS synthesis).

6.2.2 Pot experiment

The top soil was air dried and finely sieved, soil was mixed with 10% sand by volume to aid drainage. Soil was sterilized (134^o C for 4 min in a BMM Weston autoclave) and amended with 600 mg Zn kg^{-1} in the form of $ZnSO_4$, ZnS and ZnO nanoparticles in powder form. The spiked soil sample was mixed by hand for 1 hr for even distribution. Each 2.15 L pot was filled with 1 kg of spiked ($ZnSO_4$, ZnO and ZnS) and un-spiked soil (control). The experimental design (Table 6.1) contained 12 treatments with each treatment replicated in three pots, and were randomly distributed in the greenhouse. Deionised water was added to the spiked and control pots and pots were placed in individual trays to capture drained leachate throughout the experiment and were left to equilibrate for a week in the greenhouse. It is worth noting that the soil used in the glasshouse experiment was sterilized, however the glasshouse was a non-sterile environment.

Following the dose-response testing of *Rhizobium legumniosarum (bv) trifolii* and *Pseudomonas brassicacearum* (Chapter 4) to Zn, these PGPB were selected for their tolerance to zinc and their demonstrated ability to promote growth of *Brassica juncea* (Adediran et al., 2015). The selected plant growth promoting bacteria strains (*R. leguminosarum and P. brassicacearum*) were grown in a nutrient medium (containing 1 g meat extract, 2 g yeast extract, 5 g peptone, 5 g NaCl, pH 7.4) for 2 days before harvesting, centrifuged, and washed with sterile deionised water. The pelleted cells was re-suspended in sterile water to 10^8 CFU /ml.

Table 6.1 Description of experimental treatments

Codes	**Treatments**
Control	Control without Zn treatment
$ZnSO_4$	*B. juncea* grown on $ZnSO_4$ soil
ZnS nanoparticles	*B. juncea* grown on ZnS nanoparticles soil
ZnO nanoparticles	*B. juncea* grown on ZnO nanoparticles soil
B1 Control	*R. leguminosarum* control without Zn treatment
B1 $ZnSO_4$	*R. leguminosarum* + *B. juncea* grown on $ZnSO_4$ soil
B1 ZnS nanoparticles	*R. leguminosarum + B. juncea* grown on ZnS *nanoparticles soil*
B1 ZnO nanoparticles	*R. leguminosarum* + *B. juncea* grown on ZnO *nanoparticles soil*
B2 Control	*P.brassicacearum* Control without Zn treatment
B2 $ZnSO_4$	*P.brassicacearum* + *B. juncea* grown on $ZnSO_4$ soil
B2 ZnS nanoparticles	*P.brassicacearum + B. juncea* grown on ZnS *nanoparticles soil*
B2 ZnO nanoparticles	*P.brassicacearum* + *B. juncea* grown on ZnO *nanoparticles soil*

Prior to inoculation, seeds of *Brassica juncea* (L.) Czern (purchased from sow seeds Ltd., UK) were surface sterilized with 5 % NaClO for 15 minutes and washed three times with sterile distilled water under a laminar flow hood. Seeds were soaked for 4 hours in 10 ml bacteria suspension and uninoculated seeds were soaked in sterilised deionized water over the same duration before sowing in the pots. 5 seeds were sown into both spiked and unspiked (inoculated and unioculated) pots. Emergent seedlings were thinned out to a desired population density (3 plants per

pot) at 12 days after planting. Pots were individually irrigated with tap water from the tray twice a week throughout the experiment. The pot experiment was conducted in a greenhouse set to provide a day/night temperature of 21 °C in a 18 h photoperiod at a photosynthetic photon flux density (PPFD) of 150 µmol m^{-2} s^{-1} provided by cool white fluorescent bulbs. Metal-related phytotoxicity in each planted pot was evaluated by monitoring growth rate based on (i) plant height, (ii) dry biomass (iii) other observations such as leaf chlorosis and necrosis. The planted pots were harvested after 6 weeks of growth.

6.2.3 Plant sampling, Zn phytoremediation and bioaccumulation analysis.

All plants grown in both Zn spiked and unspiked soils were harvested after 6 weeks of growth. Shoots were cut 2 cm above the soil surface and washed with running tap water. Pots were emptied and roots were separated and washed in water to remove soil particles stuck on the root surface. The harvested plant material (roots and shoots) were dried in an oven to constant weight at 65 ^{o}C for 72 hours. After cooling dried samples were weighed to enable biomass calculation. Dried samples were finely ground to a homogenous sample and stored in a polyethylene tube prior to digestion for analysis. Total Zn concentrations in sub-samples of the ground plant materials and soil were determined as described in Chapter 3. Both plant extracts and soil digests were analysed for total Zn using ICP-OES (Perkin Elmer Optima 5300 DV ICP-OES). Blanks from both digests were deducted from the analytical result. Both results were reported as the mean of two sub samples of each material.

The resulting Zn concentrations from plant and soil analysis were used to evaluate Zn phytoextraction by *Brassica juncea* (L.) Czern. The following parameters were evaluated bioaccumulation factor, translocation factor and phytoextraction efficiency as detailed in Chapter 3.

6.2.4 Transmission electron microscopy (TEM) observations

TEM was used to ascertain metal accumulation in plants (Chen et al., 2007; Wilson and Bacic, 2012; Guzman et al., 2014). TEM was used in this research to map out the distribution pattern of Zn in the roots of *Brassica juncea* plants. Detailed description of TEM for inoculated and uninoculted roots of Brassica juncea (L.) Czern from different Zn treatments is presented in Chapter 3.

6.2.5 Synchroton based X-ray spectroscopic analysis

A separate pot experiment was conducted for X-ray absorption spectroscopy. The same method for growth experiment was followed, which also provided growth replication data. Live plants were used in order to avoid sample treatments such as freezing and, drying that could alter Zn speciation. Live plants were transported for mapping on microfocus beamline I18 at the Diamond light source, UK. X-ray absorption spectroscopy (XAS), XANES and EXAFS were collected at beamline I18 at the Diamond Light Source, UK as described in Chapter 3. The XRF spectra collected in Beamline I18 only were analysed (including background subtraction and peak fitting) using PyMCA 4.4.1 software (Sole et al., 2007). LCF (linear combination) was used to identify the relative proportions of Zn reference spectra within the data of samples. Linear Combination Fitting (LCF) was processed in Athena IFFEFIT software package (Ravel and Newville 2005).

6.3 Statistical analysis

All treatments were replicated three times and the means and standard error (SE) of plant height, shoot and root biomass and metal content were calculated on Microsoft Excel 2013. All treatment means were normally distributed using Anderson-Darling's normality test. The comparison of inoculated and uninoculated treatments of plant height, root and shoot biomass were compared between treatments using two-way analysis of variance (ANOVA) using Minitab software version 17 (Minitab ^{TM} Inc, State College, PA, USA). Tukey's HSD test was used to identify significant differences between treatment means.

6.4 Results

6.4.1 Growth parameters under different Zn and PGPB treatments

Figure 6.1 shows plant growth data at 6 weeks of *Brassica juncea* growth for both inoculated and uninoculated pots. Measurement of plant height started 1 week after sowing of seeds, but the result shown in Figure 6.1a is for the last measurement at 6 weeks.

Plant height was significantly lower in soil amended with ZnO NPs across all bacterial treatments, compared to the no added Zn and $ZnSO_4$ and ZnS NPs treatments. However, the uninoculated soluble Zn ($ZnSO_4.7H_2O$) treatment appeared to be the most phytotoxic as the Brassica juncea (L.) Czern. plants showed visible symptoms of toxicity (yellowing of leaves). These symptoms became more severe with increasing exposure time as the leaves of the plants began to wilt and fall off after 6 weeks of growth. There were no symptoms of toxicity in plants grown in soil amended with ZnS and ZnO NPs throughout the experiment. Inoculation with either *Rhizobum* or *Pseudomonas* bacteria resulted in significantly greater plant height across all Zn treatments compared to uninoculated controls. There is some evidence that bacterial inoculation partially ameliorated the effect of Zn addition on plant height, particularly for the $ZnSO_4$ treatment. Mean plant height grown from inoculated seeds in $ZnSO_4$-amended soil after 6 weeks (B1 $ZnSO_4$ = 37 cm and B2 $ZnSO_4$ = 36 cm) was slightly higher, although not significantly different, from the mean height of uninoculated control plants (33 cm) grown in the absence of Zn contamination.

For shoot dry biomass significant differences were identified between the combined effect of Zn and inoculation treatments, indicated by the different letters in Figure 6.1b. In the absence of bacterial inoculation, shoot dry biomass after 6 weeks was significantly higher in the control treatment with no added Zn compared to the treatments with added Zn. However, bacterial inoculation resulted in the recovery of shoot dry biomass when Zn contamination is present in the form of $ZnSO_4$, so that it was not significantly different from that of the uninoculated control treatment with no added Zn after 6 weeks. This effect of bacterial inoculation was

not observed in the Zn NPs treatments, in which shoot dry biomass in the inoculated ZnO and ZnS treatments was significantly lower than the uninoculated control with no added Zn.

For root dry biomass significant differences were identified between the combined effect of Zn and inoculation treatments, indicated by the different letters in Figure 6.1c. Root biomass was significantly lower in the $ZnSO_4$ treatments compared to the controls in both inoculated and uninoculated treatments. However, inoculation with *Pseudomonas* overcame some of the differences in root biomass between the Zn amended treatments and the no added Zn control, with no significant difference in root biomass between that of the control and the $ZnSO_4$ and ZnS NPs treatments. In contrast, inoculation with *Rhizobium* appeared to promote root biomass only in the ZnS NPs treatment, with significantly lower root biomass occurring in the $ZnSO_4$ and ZnO NPs treatment compared to the no added Zn control.

Overall, in the absence of inoculation with PGPBs, addition of any of the Zn species investigated had a detrimental effect on plant growth, although visual symptoms of toxicity suggested that $ZnSO_4$ was the Zn species which had the greatest adverse effects on plant growth in the absence of PGPB inoculation. Inoculation with PGPB alleviated, to some extent, the effect of Zn exposure on plant growth. In all the inoculated Zn treatments, plants appeared healthy and visible signs of Zn toxicity were absent. Growth of *Brassica juncea* following bacteria inoculation was enhanced more in $ZnSO_4$ than in the ZnS and ZnO NPs treatments as indicated by no significant differences in shoot dry biomass between the inoculated $ZnSO_4$ treatments and the uninoculated control with no added Zn.

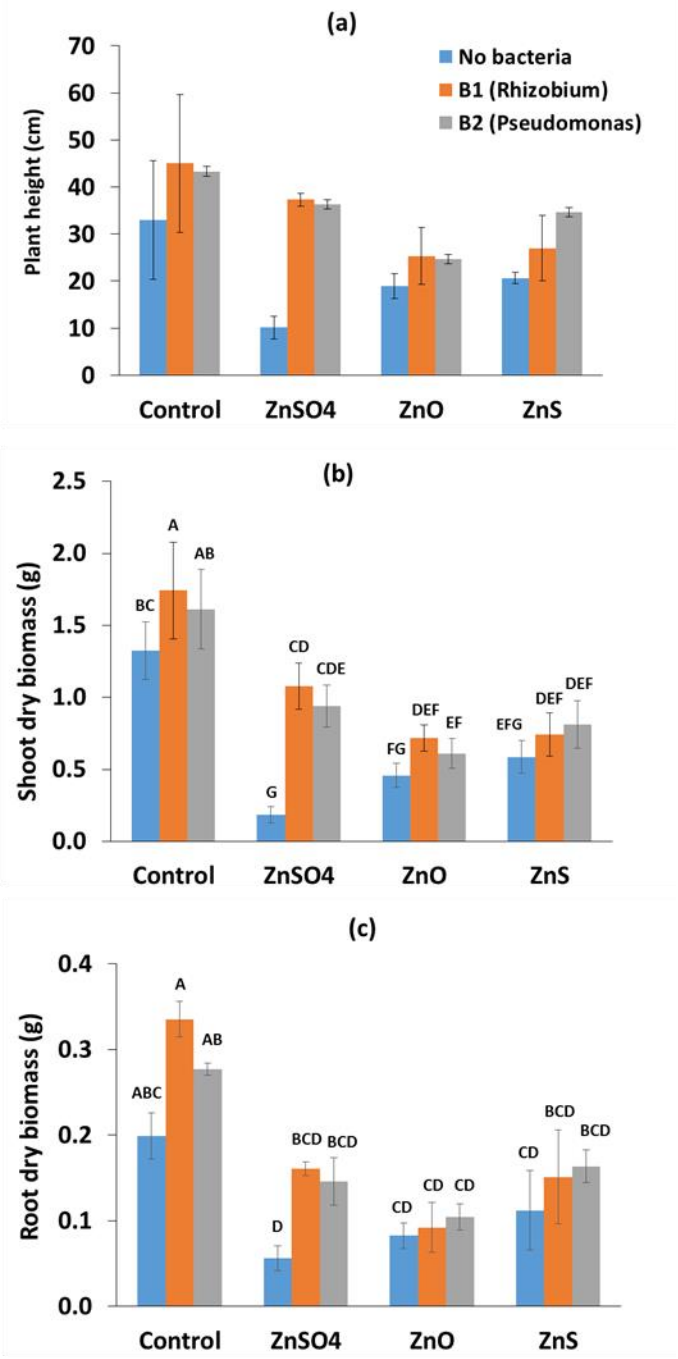


Figure 6.1: Effect of PGPBs on plant height and biomass after 6 weeks of growth in 600 mg Zn kg^{-1} Zn treatments. (a) Inoculated and uninoculated plant height (b) inoculated and uninoculated shoot biomass, (c) inoculated and uninoculated root biomass. B1 represent *Rhizobium leguminosarum* and B2 represent *Pseudomonas brassicacearum.* Bars are means and error bars represent on means of three pots. Significant differences are indicated by different letters at $p < 0.05$ following Tukey multiple comparison tests.

6.4.2 Effect of PGPB on Zn accumulation

PGPB effect on Zn uptake was evaluated in roots and shoots of *Brassica juncea* (Figure 6.2). Result showed variations in Zn contents in shoot and roots of *Brassica juncea* for different Zn treatments (Figure 6.2 a-b). Zn concentration in shoots was considerably higher than in the roots for both inoculated or uninoculated treatments as seen in Figure 6.2, consistent with *Brassica juncea* being a Zn phytoextractor. However, inoculation with *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* enhanced Zn concentration in roots and shoots of plants grown in $ZnSO_4$ than in ZnO and ZnS nanoparticles treatments indicating that both PGPBs assisted in enhancing soluble Zn uptake in *Brassica juncea.* Zn concentration in root and shoot biomass increased in the order of $ZnSO_4$ > ZnO > ZnS > control.

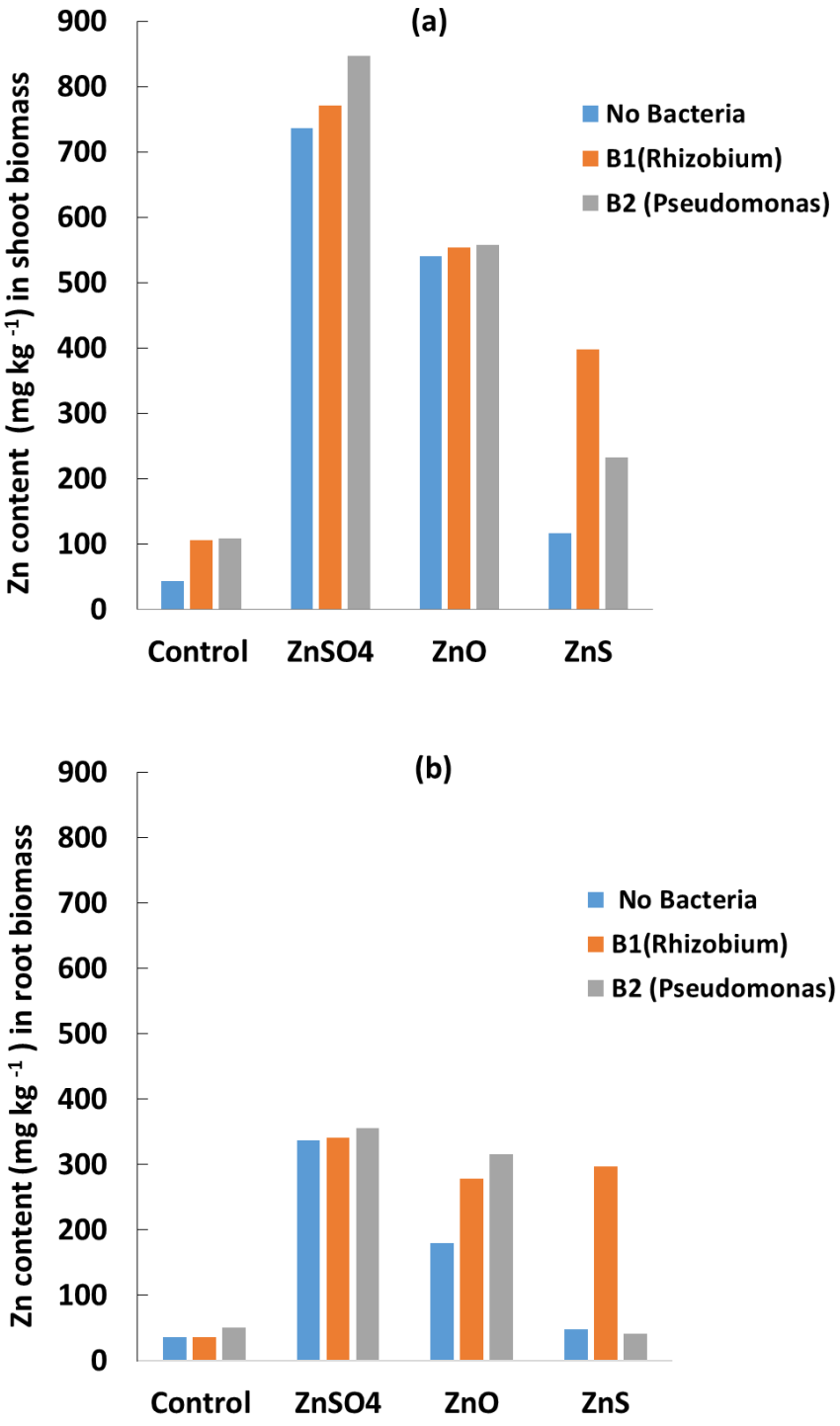


Figure 6.2: Zn concentrations in (a) inoculated and uninoculated shoot biomass, (b) inoculated and uninoculated root biomass 6 weeks after planting in in 600 mg Zn kg^{-1} treatments. B1 represent *Rhizobium leguminosarum* and B2 represent *Pseudomonas brassicacearum*. Values are means of two sub samples of each treatment.

6.4.3 Evaluation of effects of PGPBs on Zn uptake and phytoextraction

Bioaccumulation (BCF) and translocation factors (TF) were calculated in inoculated and uninoculated plants under different Zn treatments (Table 6.2). BCF values were > 1, indicating Zn bioaccumulation in plant tissue compared to soil, for all Zn treatments except for ZnS NPs treatments with no bacteria and with *Pseudomonas brassicacearum* (B2) inoculation. Overall, however, values of BCF were higher in the inoculated than uninoculated treatments for all Zn species. The TF values, the ratio of the shoot to root Zn concentrations, were > 1 in the inoculated and uninoculated treatments for all Zn species, but the response of TF values when plants were inoculated varied between the different Zn species treatments. TF values increased slightly in inoculated plants growing in $ZnSO_4$ contaminated soils, compared to uninoculated plants. The opposite response occurred in ZnO NPs contaminated soils, with lower TF values occurring in the inoculated compared to the uninoculated plants. In the ZnS NPs contaminated soils, compared to inoculated plants, the TF value also decreased in plants inoculated with *Rhizobium leguminosarum* (B1) but increased in plants inoculated with *Pseudomonas brassicacearum* (B2).

Zn mass removal by *Brassica juncea* was estimated to compare the phytoextraction efficiency (PE) of Zn by inoculated and uninoculated plants from soil contaminated with different Zn species after 6 weeks of plant growth. PE values for Zn removal by *Brassica juncea* were higher in the inoculated plants compared to uninoculated plants in all Zn species treatments (Table 6.2). Zn removal efficiency was very low in all 600 mg kg^{-1} Zn treatments (<0.3% of soil Zn concentration), although PE values were an order of magnitude higher in inoculated plants in $ZnSO_4$-contaminated soils, compared to inoculated plants growing in the ZnO and ZnS NPs contaminated soils.

Table 6.2 Bioaccumulation factors, Translocation factors and phytoextraction efficiency in *Brasssica juncea* after 6 weeks of growth in soil amended with 600 mg Zn kg^{-1} of different Zn species and with and without inoculation with PGPB. B1 represents *Rhizobium leguminosarum* and B2 represents *Pseudomonas brassicacearum.*

Parameters	$ZnSO_4$			ZnO nanoparticles			ZnS nanoparticles		
	No bacteria	B1	B2	No bacteria	B1	B2	No bacteria	B1	B2
Bioaccumulation factor (BCF)	1.78	1.85	2.0	1.19	1.39	1.45	0.27	1.15	0.46
Translocation factor (TF)	2.18	2.25	2.38	3.01	1.99	1.77	2.43	1.33	5.54
Phytoextraction efficency (PE, %)	0.05	0.28	0.26	0.04	0.07	0.06	0.01	0.04	0.03

6.4.4 Distribution of Zn in *Brassica juncea* biomass

Due to similar growth of plants inoculated with the two different strains of PGPB, only plants inoculated with *Pseudomonas brassicacearum* were selected for comparison with uninoculated plants for all Zn species treatments using transmission electron microscopy (TEM) (Figure 6.3). Micrographs indicated differences in the morphology and location of Zn in roots of *Brassica juncea* following different Zn exposures. Black particles or dense dots could be seen deposited in different parts of the root cell. Roughly spherical Zn nanoparticles on the epidermis and root surfaces in ZnO nanoparticles treatment (Figure 6.3B) were observed. In the roots of inoculated *Brassica juncea* plants, less bacteria were evident in the Zn NPs treatments (Figure 6.3E-F) compared to the $ZnSO_4$ treatment, where they occurred around the root epidermis (Figure 6.3D).

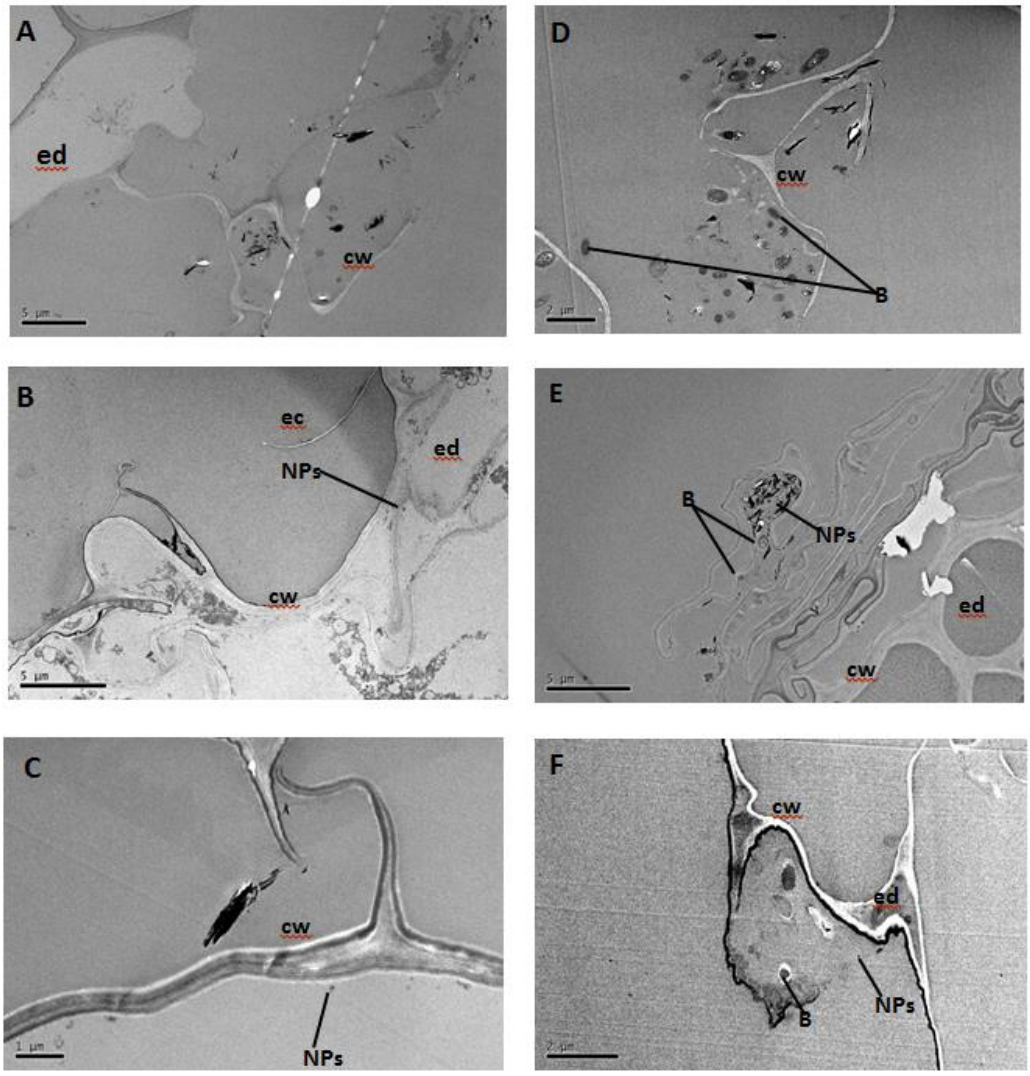


Figure 6.3: TEM micrograph of a cross section (bar 1-5 µm) of (A-C) uninoculated $ZnSO_4$, ZnO NPs and ZnS NPs and (D-F) inoculated root cells of *Brassica juncea* exposed to 600 mg kg $ZnSO_4$, ZnO NPs and ZnS NPs, after 6 weeks of growth. Arrows in the root cell indicate: NPs- nanoparticles, cw- cell wall, ed-endodermis, ec- epidermic cell, B- *Pseudomonas brassicacearum*

6.4.5 In situ speciation of Zn in *Brassica juncea* roots

Zn distributions in roots of *Brassica juncea* were investigated using µ-XRF maps and results are shown in Figure 6.4. The results shown below focus on live roots exposed to $ZnSO_4$, ZnO and ZnS nanoparticles for comparison and also as a function of bacteria inoculation. Due to limited time on the beamline, only roots inoculated with *Pseudomonas brassicacerum* (B2) were collected, chosen because this bacterium is known to be endophytic to *Brassica juncea*. The pixel brightness is displayed in RGB, and the different colours represent relative concentration of Zn from high (red) to low (blue), after normalisation of the fluorescence counts to background, allowing relative spatial concentration distributions to be compared. Data are presented as intensity maps that allow for the spatial distribution of Zn to be shown.

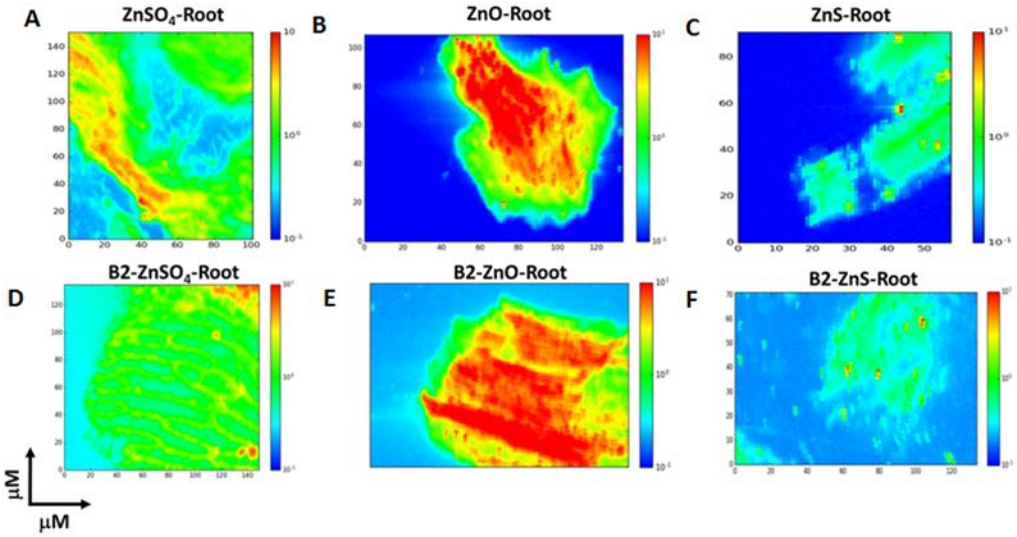


Figure 6.4: Synchrotron µ-XRF mapping of the transversal section of live roots from (A-C) uninoculated and (D-F) inoculated *Brassica juncea* plant grown in soil treated with 600 mg Zn kg^{-1} of $ZnSO_4$, ZnO and ZnS nanoparticles. µ-XRF map showing Zn distribution with pixel brightness displayed in RGB, red represents higher Zn intensity, and the blue represents absence of Zn signal in the uninoculated and inoculated Zn treatments.

The intensity of Zn as exhibited by the tricolor maps indicate Zn was differently distributed within the roots of *Brassica juncea* for all Zn treatments. The highest relative background concentrations were recorded in the $ZnSO_4$ treatments (Figure 6.4A, D). In these treatments localised Zn hotspots were evident but the most distinctive characteristic was that high Zn concentrations above background occurred in the form of stripes. In ZnO treatments (Figure 6.4B, E), background concentrations were also high (close to those of $ZnSO_4$) and significantly, the endodermis (red colour) exhibited Zn concentrations that were about an order of magnitude higher than background. Single hotspots of high Zn concentration were also evident. ZnS treatments showed the lowest background concentrations with high Zn concentrations only occurring as single hotspots (Figure 6.4C, F). Hotspots of Zn in the roots treated with ZnO and ZnS nanoparticles may indicate the presence of Zn nanoparticles in the roots of *Brassica juncea*. Comparison between inoculated (Figure 6.4D-F), and uninoculated (Figure 6.4A-C) plants showed no significant impact of bacteria inoculation on Zn concentrations in the root in each treatment, and thus the differences appear to reflects initial Zn speciation.

6.4.6 Speciation of Zn in *Brassica juncea* plants by XANES

Zn μXANES spectra were acquired on a few of the Zn hot spots to provide information on the chemical froms in which zinc is present in the roots. To obtain quantitative information of Zn speciation, XANES spectra were interpreted using linear combination fiting of spectra from selected Zn standards listed in Table 3.6 (Chapter 3). LCF calculated the contribution of a range of reference (standards) compounds run under identical conditions to XAFS signal of the sample. The goodness of the fit was estimated by determining the residual R factor between the root sample fits and in combination with the Zn standard fits. The best fits based on residual R factors are presented in Figure 6.5 a-c.

Fitting the $ZnSO_4$ suggested Zn was in the form of Zn Histidine (52 %), Zn phytate (39 %), and Zn carbonate (12 %) in the root. Upon inoculation with *Pseudomonas*

brassicacearum, roots treated with $ZnSO_4$ suggested Zn was in the form of Zn cysteine (63%), Zn sulfate (25 %), and Zn carbonate (17 %).

Fitting of ZnO nanoparticles data suggested that Zn existed mostly as Zn cysteine (55%), Zn histidine (36%) and Zn phytate (8%), while the inoculated roots indicated Zn was in the form of Zn cysteine (68%), Zn histidine (16%) and Zn phytate (20%).

ZnS nanoparticles spectra were best fitted with Zn in the form of Zn cysteine (51 %), Zn carbonate (30 %) and Zn sulfate (11%). Upon inoculation with *Pseudomonas brassicacearum*, roots treated with ZnS nanoparticles suggested Zn was in the form of Zn cysteine (55%), Zn PGA (22 %), and Zn carbonate (17 %). This indicates that inoculation with *Pseudomonas brassicacearum* changed Zn speciation in plant roots exposed to different Zn species.

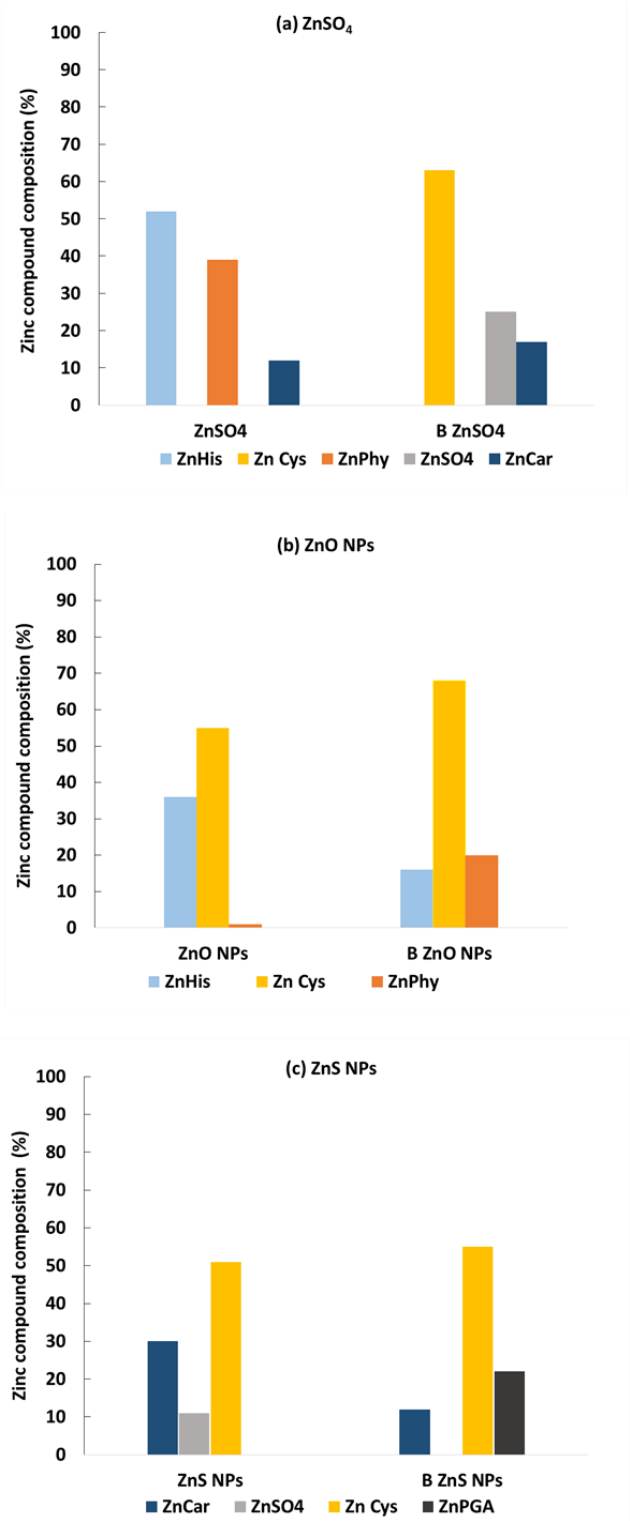


Figure 6.5: Linear combination fitting of $ZnSO_4$, ZnO and ZnS data from hot spots of Zn microXRF mapping. Bar charts represent contribution (%) of the various species to the spectra of uninoculated and inouclated roots of (a) $ZnSO_4$, (b) ZnO and (c) ZnS nanoparticles treaments. Zn standards are: ZnCarb- Zn carbonate, ZnPhy- Zn phytate, ZnHis- Zn histidine, $ZnSO_4$- Zn sulphate and ZnCys- Zn cysteine.

6.5 Discussion

Inoculation with PGPB was examined in order to assess their phytoremediation potential in Zn contaminated soil. A critical understanding of the role of PGPB was sought based on comparison between soluble Zn and Zn nanoparticles in plant growth, uptake and distribution of Zn in *Brassica juncea* as discussed below.

6.5.1 Effects of bacteria on Zn toxicity and accumulation from different Zn species

Brassica juncea grew in Zn contaminated soil but with significant differences in plant productivity linked to different Zn forms applied in soil. Of all Zn treatments used in this study, uninoculated plants exposed to $ZnSO_4$ were more adversely affected than the Zn nanoparticles (Figure 6.1). Given that $ZnSO_4$ is the most soluble of the forms used and the solubility product constants for ZnO and ZnS nanoparticles are about 10^{-17} $(mol/L)^3$ and 10^{-23} $(mol/L)^2$ (Mudunkotuwa et al., 2011; Rupasinghe, 2011) respectively, observed toxicity trends are entirely consistent with solubility being the primary control on toxicity of Zn to *Brassica juncea*.

On the contrary, our result showed significant increases in plant height, and biomass, indicating plants adapted to Zn stress in soils more effectively upon inoculation with *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* than in uninoculated treatments (Figure 6.1). These positive effects on *Brassica juncea* growth in Zn contaminated soil are similar to the findings of Adediran et al. (2015), although that study was limited to $ZnSO_4$ treatment and also used a different growth medium. The potential for *R. leguminosarum* and *P. brassicacearum* to enhance growth parameters in inoculated *Brassica juncea* plants may be attributed to the PGPB traits including the production of indole acetic acid (IAA), ACC deaminase, solubilisation of phosphate, production of siderophores (amongst others) (Khan et al., 2009; Rajkumar et al., 2010; Ahemed and Kibret, 2014; Ma et al., 2015a, b). However, these PGPB properties were not examined in this study so will not be discussed further.

Accumulation of high Zn concentration in shoot than in the roots (Figure 6.2) regardless of presence of PGPBs indicated an effective translocation of Zn from roots to shoots for all Zn treatments. However, the inoculated Zn treatments had higher Zn concentration in plant tissue than in uninoculated Zn treatments, with $ZnSO_4$ exposed plants accumulating more than the Zn nanoparticles. The higher Zn concentration in inoculated plant biomass (Figure 6.2) may be attributed to PGPBs role in increasing Zn concentrations in soil by altering the solubility and availability of Zn. Sessitsch et al., (2013) reported that PGPB can induce acidification of rhizospheric soils of plants by producing organic acids, which enhance metal bioavailability around the root zone and hence plant metal uptake. Although pH was not measured in this pot experiment, we show later (Chapter 7) that the presence of these PGPB did lead to a decrease in pH in the soil, suggesting it may be a possible mechanism. In any case, our findings are similar to other studies that have reported that inoculation with PGPBs increases plant growth, metal uptake, tolerance and phytoremediation process in contaminated soils (Rajkumar et al., 2008; Zhang et al., 2011a, b). On the contrary, other studies have reported that PGPB inoculated plants increase growth and tolerance but reduce metal uptake (Rajkumar et al., 2013). This suggest that different PGPBs elicit different responses that may also depend on the hyperaccumulator species (Ma et al., 2015a, b).

Brassica juncea ability to uptake metal from soil was evaluated using bioaaccumulator factors (BCF) and translocation factors (TF) (Brunetti et al., 2009). BCF is the total metal concentration in plant to metal concentration in soil. TF is the plant ability to absorb and translocate metal into aboveground plant part (Marchiol et al., 2004). Plants are considered as potential species for phytoextraction if both BCF and TF are greater than one (Ahmad et al, 2011). In this study, bioaccumulation (BCF) and translocation factors (TF) varied with different Zn exposure. BCF was >1 for inoculated and uninoculated $ZnSO_4$ and ZnO nanoparticles treatments than in ZnS nanoparticles treatment except for plants inoculated with *Rhizobium leguminosarum* only (Table 6.2). The TF values were >1 for all inoculated and uninoculated Zn treatments, however indicating that Zn was translocated from root

to shoots. Thus *Brassica juncea* exhibited Zn hyperaccumulative characteristics, with bacteria enhancing these characteristics. However, the overall phytoremediation potential was extremely low, with a maximum of 0.28% Zn extracted from $ZnSO_4$ treated in the presence of bacteria (Table 6.2).

6.5.2 Distribution and speciation of Zn in plant roots

Many hyperaccumulating species exposed to toxic concentration of metal ions reduce or prevent metal uptake through metal exclusion or cytosolic chelation and vacuolar sequestration (Clemens, 2010; Sun et al., 2011; Manara, 2012). In this study, XAS and TEM mapping and analysis was used to investigate mechanisms of high Zn tolerance in the presence of bacterial inoculation. The observed spatial distribution of Zn in the roots of *Brassica juncea* examined using synchrotron μ- XRF and TEM suggested that hyperaccumulation of Zn in *Brassica juncea* is dependent on the form of Zn spiked in soil. TEM confirmed variation in root Zn accumulation in *Brassica juncea* (Figure 6.3) highlighting differences in Zn distribution of $ZnSO_4$ and nanoparticulate Zn. Some studies have reported that cellular penetration by nanoparticles is the mode of action by which nanoparticles can interact with plants (Lin and Xing, 2008; Lin et al., 2009; Chen et al., 2010). We speculate that the dense black dots or clusters observed in the root epidermis and intracellular spaces in Zn nanoparticles treated roots are Zn nanoparticles suggesting that *Brassica juncea* absorbed these Zn nanoparticles, but this remains an open question at the moment, because we did not have analytical capability on the TEM to check the composition. Once inside a plant cell, nanoparticles can be transported apoplastically or symplastically through plasmodesmata (Lucas and Lee, 2004; Zhang et al., 2015). Substantial proof of the presence of *P. brassicacearum* was evident in roots exposed to Zn treatments (Figure 6.3 D-F) suggesting that *P. brassicacearum* formed a close association with plant roots by colonising the roots thus may be inducing plant resistance to toxic Zn and promoting plant growth. *P. brassicacearum* is originally isolated from the rhizoplane of plants belonging to the Brassicaceae (*Brassica napus and A. thaliana*) (Achouak et al., 2000), has known capability of colonizing root areas (Long et al., 2008) and exhibiting plant growth promoting

properties (Ortet et al., 2011). High spatial resolution, synchrotron based X-ray fluorescence mapping provided complementary information to TEM into the cellular distribution of Zn in the roots of *Brassica juncea* (Figure 6.4). μXRF Zn hotspots were less prevalent in roots exposed to ZnS than roots exposed to ZnO nanoparticles and $ZnSO_4$ treatments, as also confirmed by the TEM (Figure 6.4). This may be attributed to the less Zn concentration in plant tissue treated with ZnS nanoparticles (Figure 6.5).

Following the spatial imaging of Zn in roots in different Zn treatments, LCF was essential in investigating the speciation of Zn in the root samples. Linear combination fitting strongly showed that Zn was predominantly bound to Zn Histidine in uninoculated plant roots treated with $ZnSO_4$ treatments with large percentage differences between phytate and carbonate in uninoculated $ZnSO_4$ treatment (Figure 6.5 a), suggesting that excessive Zn in plants treated with $ZnSO_4$ may have been limited by the presence of Zn histidine in the roots. Such complexation with high affinity ligand (Histidine) would likely reduce the toxicity of Zn to the plant (Salt et al., 1999; Clemen, 2006), since complexed metals tend to be less bioavailable compared to uncomplexed metals (Erten-Unal et al., 1998). Histidine as an important amino acid and has been shown to bind metals in hyperaccumulator species (Kramer et al., 1996) including Zn (Salt et al., 1995; Kupper et al., 2004; Leitenmaier and Kupper, 2013). In addition, the presence of phytate in $ZnSO_4$ roots after 6 weeks of plant growth may be regulating the toxicity of Zn to plants. Zn phytate (phytate myoinositol hexakisphospahte) is a more complex phosphate containing molecule with a negatively charged phosphate group that forms stable complexes with ions including Zn^{2+} (Raboy et al., 2001; Kopittke et al., 2011; Lv et al., 2015). The presence of Zn phytate in roots has been suggested as a Zn tolerance mechanism in non- hyperaccumulating plants (Sarret et al., 2002, Terzano et al., 2008), and recently Zn phytate was indicated in *Brassica juncea* (Adediran et al., 2016), as a possible mechanism for Zn tolerance.

Whilst histidine is important in uninoculated roots treated with $ZnSO_4$, inoculation with *Pseudomonas brassicacearum* entirely changed Zn speciation in roots of the

$ZnSO_4$ treatment (Figure 6.5 a). Zn histidne, and phytate were completely replaced with significant high proportion of Zn cysteine with lesser Zn associated with sulfate and carbonate (Figure 6.5a). This change following inoculation with *Pseudomonas brassicacearum* may reveal bacterial enhanced cysteine production in the roots of *Brassica juncea* (Adediran et al., 2016).

The speciation of Zn in nanoparticulate treatments differed markedly from $ZnSO_4$ treatments (Figure 6.5 b-c). The speciation of Zn was the same for uninoculated and inoculated roots in ZnO NP treatments. However, the proportion of Zn cysteine was higher for the inoculated treatment (Figure 6.5 b). The high proportion of cysteine detected in the roots of *Brassica juncea* exposed to ZnO NPs where no sulphur was supplied was unexpected. However, the sulphur content (248.7 mg/kg) in the study soil may be conducive to cysteine synthesis even in the presence *Pseudomonas brassicacearum.* Although Zn was primarily associated with cysteine in ZnO treatment, lesser Zn was associated with Zn phytate and Histidine. Zn bound to phytate in ZnO treatments is likely, since ZnO may undergo biotransformation to form Zn phosphate (mainly as Zn phytate) (Terzano et al., 2008; Lv et al., 2015) or Zn may have been precipitated in roots as Zn phosphate thus limiting zinc bioavailability (Lee et al., 1996).

In ZnS NP treatments, Zn cysteine was also the predominant form of Zn. Zn cysteine accounted for the highest proportion of zinc in roots exposed to ZnS treatment, with 55% of cysteine found in inoculated roots attributing to better Zn tolerance in roots of *Brassica juncea*. Besides, Zn cysteine, small proportion of Zn associated with the polygalacturonic acid (PGA) in inoculated roots (Figure 6.5 c). The walls of root cells are directly exposed to Zn in soil, cell wall associated Zn is bound to PGA (Singh, 2005). Complexation of zinc with carboxylic acids such as polygalacturonic acids (the main component of pectin in the cell wall) has been reported as a response mechnanism to metal toxicity in plants exposed to high concentration of zinc (Kopittke et al., 2011; Dalvi and Bhalerao, 2013; Adediran et al., 2016).

The LCF in this study showed a complete absence of ZnO and ZnS nanoparticles in plant roots of *Brassica juncea*. This may suggest that the majority of Zn nanoparticles were taken up as NPs (hence as they appeared as spots in Figure 6.4) but once inside, NPs are dissolved and converted to other species, or they are compartmentalised or limited in transport to diffusion. It is assumed that nanoparticulate phases must be dissolved before Zn can be taken up by plants (Hernandez-Viezcas et al., 2013). A few recent studies have reported the absence of nanoparticulate ZnO in plants challenged with ZnO nanoparticles instead Zn was in the form of nitrates, citrate and phosphates (Lopez- Moreno et al., 2010; Hernandez-Viezcas et al., 2011; Hernandez-Viezcas et al., 2013). However, other studies have reported internalisation of ZnO nanoparticles in different plants (Dimkpa et al., 2013). Wang et al. (2013) used synchrotron based techniques to compare the transformation of ZnO nanoparticles with soluble ZnCl in plant roots of cowpea (*Vigna unguiculata*). The authors reported that ZnO nanoparticles underwent rapid dissolution in soil, with Zn associated with citrate, histidine and phytate but in solution culture, Zn was largely associated with ZnO nanoparticles (65%) and histidine (38%). The lack of ZnS and ZnO species in Zn nanoparticle exposed roots, may suggest that both ZnO and ZnS must be dissolved before entering the root as dissolved Zn ions. It also appears that whether nanoparticles are taken up by plants depends on the type of nanoparticles, growth medium and the plant species involved (Gardea-Toresdey et al., 2014).

Zn cysteine was the most dominant form of Zn associated with all three Zn treatments with high proportion observed in the inoculated root samples. Zn is mainly associated with the cysteine due to strong binding of Zn to S. Cysteine-rich polypeptides (Metallothiones and phytochelatins) are intracellular ligands capable of coordinating Zn ions through the thiol groups of their cys residues (Rauser, 1995; Cobbet, 2000; Kinraide, 2009; Zeng et al., 2011; Hossain et al., 2012). The presence of cysteine- bound zinc as identified in the roots of *Brassica juncea* in the different Zn treatments may suggest that the Zn treatments induced cysteine synthesis. However, it is recognised that plants assimilate inorganic sulphur as sulfate from the soil which is reduced to sulphide and incorporated into cysteine (Romero et al.,

2014). Cysteine biosynthesis in plant begins with the formation of O-acetylserine from acetyl- CoA and serine catalysed by serine acetyltransferase (SAT) (Takahashi et al., 2011; Yi et al., 2013). O-acetylserine (thiol) lyase (OASTL) uses pyridoxal-5'-phosphate as cofactor to generate cysteine from O-acetylserine and sulphide (Jez et al., 2016). These two enzymes (SAT and OASTL) are known to catalyse the formation of cysteine in both plants and bacteria (Leustek et al., 2000; Bonner et al., 2005; Jez and Dey, 2013). Since cysteine is the main sulphur source in plant roots, sulphur metabolism in response to Zn stress suggests that cysteine or a downstream metabolite, such as glutathione, may play a role in Zn tolerance and detoxification (Zeng et al., 2011; Ma et al., 2015). In all Zn treatments, *P. brassicacearum* inoculated plants led to a significant improvement in plant growth due to the presence of high proportion of Zn cysteine. Thus, this study suggest an additional detoxification mechanism associated to bacteria-enhanced synthesis of cysteine in the presence of *Pseudomonas brassicacearum* as a key role in ameliorating Zn toxicity in Zn contaminated soil.

6.6 Conclusion

Zinc accumulation and distribution in hyperaccumulator species are vital in elucidating the role of plant growth promoting bacteria in remediation of Zn contaminated soil. The result of the present study revealed that inoculation of Zn resistant *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* increases the phytoremediation efficiency of Zn by enhancing Zn accumulation in plant tissue and by promoting plant growth than in control treatments. This work suggested different uptake mechanisms for $ZnSO_4$, ZnO and ZnS nanoparticles in *Brassica juncea*. The result herein, highlights inoculation with *Pseudomonas brassicacearum* demonstrated considerable increase in tolerance and detoxification of toxic Zn in *Brassica juncea* and subsequently improved plant growth and development which is mediated by presence of cysteine as a possible mechanism to plant response in Zn contaminated soil. It also appears that bacteria do not only induce speciation changes in plant tissues but may also act by influencing the amount of bioavailable Zn in soil, resulting in efficiently resisting Zn toxicity. Whether or not this mechanism on the role of PGPB in plant uptake and in ameliorating Zn toxicity is attributed to speciation changes in plant roots or speciation changes in soil-rhizospheres- plant interface remains unknown but however, may merit further investigation in the next Chapter 7.

Chapter 7

7 Role of plant growth promoting bacteria in promoting metal speciation gradients across soil-rhizosphere-plant interfaces in metal contaminated soils

7.1 Introduction

Metals and metal nanoparticles are continuously being added to soils resulting in negative effects on the entire environment (Khan, 2005; Khan et al., 2008; Hernandez-Viezcas et al., 2011). Plant growth and survival in metal contaminated soils is dependent on the ability of plants to adapt to high metal concentrations (Chiang et al., 2006). One possible plants adaption strategy is for the roots of plants to facilitate chemical and physical changes in the adjacent soil, especially at the interface between the soil and roots (rhizosphere) which greatly differs from the surrounding bulk soils (Curl and True, 1986; Marschner, 1995; Lynch, 1990; Chiang et al., 2006; Hinsinger et al., 2009). The term "rhizosphere" describes the zone of soil around the plant root where root exudates secreted by plant roots can stimulate or inhibit soil microbial activities (Dessaux et al., 2016). This soil environment can be as thin as a few µm or as thick as a few cm (Alford et al., 2010) depending on soil type and properties, and plant species, including root system architecture and nature of exudates (Hinsinger and Courchesne, 2008). Root exudates are an important characteristic of the rhizosphere, influencing the behaviour of metals, either enhancing or reducing their availability, by directly affecting acidification, redox reactions, precipitation and chelation, or indirectly through their effects on microbial activity and root growth pattern (Tao et al., 2004; Kidd et al., 2009).

Hyperaccumulators are known for their ability for metal uptake which is dependent on metal availability or on the extent to which the rhizosphere is modified (McGrath et al., 1997; Wenzel et al., 2003; Hinsinger and Courchesne, 2008). Plant induced modification of metal speciation in the rhizosphere is the result of steep gradients

in metal concentration, pH, redox potential, pO_2, pCO_2, microbial biomass and organic ligand concentrations (Pushchenreiter et al., 2005; Kidd et al., 2009). Metals exist in different forms in soils from vey labile species to non-labile fixed forms (Dessreault-Rompre et al., 2006). Excessive metal uptake by plants has been associated with partial reduction of labile, easily bio-accessible metal fractions in the rhizosphere (Hammer and Keller, 2002; Puschenreiter et al., 2003) mediated by active root proliferation towards areas of high metal concentration (Schwartz et al., 1999).

Bacteria that are closely associated with roots are termed plant growth promoting bacteria (PGPB) (Glick, 1995) or rhizobacteria. However, their role in modifying metal speciation at the rhizosphere is not yet fully understood. The research literature to date suggests that these heterogeneous groups of free –living bacteria can significantly increase metal uptake and accumulation, promote plant growth and alleviate metal toxicity in plants (Zhuang et al., 2007; Weyens et al., 2009; Glick, 2010; Luo et al., 2012; Zheng et al., 2012; Adediran et al., 2016). Therefore, the total rhizosphere environment is governed by an interacting trinity of the soil, the plant, and the organisms associated with the root (Lynch, 1990; McNear, 2013). Understanding the interplay of metal accumulating plants with their rhizosphere microbiome is an important step towards the application and optimization of phytoremediation (Meharg and Cairney, 2000; Fitz and Wenzel, 2002; Kupper et al., 2004; Wenzel, 2009).

Studies have investigated the physiology and molecular mechanism of Zn hyperaccumulation in *Brassica juncea* (Adediran, 2015; 2016). Other studies using plants suitable for phytoremediation showed a significant reduction in plant available metal fractions in the rhizosphere. Kim et al. (2010) investigated the influence of *Brassica juncea* root exudation on soil solution properties in the rhizosphere using a rhizobox and reported that changes in pH and DOC derived from the rhizosphere are principally responsible for subsequent changes in the metal solubility. Panfilli et al. (2005) showed that grass species *Festuca rubra* (red fescue) and *Agrostis tenuis* (colonial bent grass) grown on contaminated dredged

sediment accelerated the weathering of ZnS, thus increasing Zn bioavailability in the rhizosphere. After 2 years of plant growth, µm –sized Mn-Zn black precipitates were observed on the surface of *Festuca rubra* roots which were identified as a Zn-rich phyllomanganate. From this it was suggested that Zn biomineralization by plants is likely to be a defence mechanism against metal toxicity (Lanson et al., 2008). However the mechanism involved in the root-induced speciation changes and the modification of these changes by PGPB across the soil-rhizosphere-plant interface was ignored in the study. There is a gap in knowledge of the root induced changes and the processes controlling metal contaminants in the rhizosphere of *Brassica juncea* (a Zn hyperaccumulator*)* and there is virtually no information comparing the speciation of Zn between soluble Zn ($ZnSO_4$) with Zn nanoparticles (ZnS nanoparticles) in rhizospheres of *Brassica juncea (L.) Czern.* Addressing these knowledge gaps may provide a novel insight into mechanisms of Zn hyperaccumulation for phytoextraction of Zn contaminated soil.

Thus the aims of this study are to (1) investigate root- induced speciation changes of different Zn species in the rhizosphere of *Brassica juncea (L.) Czern;* (2) determine whether such changes affect the uptake, accumulation and distribution of Zn in the plant; and (3) investigate the role of the PGPB *Rhizobium leguminosarum (bv) trifolii* in modifying speciation across the soil-rhizosphere-plant interface. It was hypothesised that: (1) the speciation changes in plant roots will not be different in the soil-rhizosphere, and (2). *Rhizobium leguminosarum (bv) trifolii* will modify speciation across the soil-rhizosphere-plant interface of *Brassica juncea (L.) Czern.*

7.2 Materials and Methods

7.2.1 Materials

This study focused on contamination by soluble Zn (in the form of $ZnSO_4$. $7H_2O$) and ZnS nanoparticles. ZnS was used as a model nanoparticle relating to pollution from mining of sphalerite. In this case, however, ZnS nanoparticles were synthesised in the laboratory by using a chemical precipitation method (Ganguly et al., 2014) as detailed description in Chapter 3. Zinc sulphate ($ZnSO_4.7H_2O$) was purchased from Sigma Aldrich, UK.

Westland topsoil was purchased from Dobbies Garden Centre Edinburgh, UK. Soil was air-dried, crushed, and passed through a 2 mm stainless steel sieve to disaggregate clumps and remove any coarse debris present. The soil was amended with 10 % sand to improve drainage. The air dried soil was sterilised by autoclaving (BMM Weston autoclave) for 35 min at 134 ^oC, and amended with 600 mg Zn kg^{-1} in the form of $ZnSO_4$, or ZnS nanoparticles. The Zn concentration chosen was sufficient to trigger toxic effects in plants without completely curtailing growth and also to ensure detection of the Zn X-ray signal in plant and soil samples during X-ray absorption spectroscopy analysis.

Brassica juncea (L.) Czern was chosen for this study. Also known as Indian mustard, it is an annual plant with a short growing season and has been identified as a hyperaccumulator because of its fast growth rate, high metal accumulation and translocation of Zn from root to shoot (Wang et al., 2009; Qu et al., 2012; Adediran et al., 2015). Seeds of *Brassica juncea* were purchased (Sow Seeds Ltd., UK) and stored in a clean plastic bag and kept in the dark at room temperature (14 -16 ^oC) until use.

7.2.2 Experimental design

The sterilized spiked soil sample was mixed by hand for 1 hr to produce a homogenous sample. Each 2.15 L plastic pot for an experimental replicate (Table 7.1) was filled with 1 kg of either spiked (Zn, or ZnS nanoparticles) or un-spiked soil (control) and were randomly distributed in the greenhouse space. The pots were placed in individual trays to capture drained leachate throughout the experiment (Kos et al., 2003; Giordani et al., 2005) and were left to equilibrate for a week in the greenhouse.

A single colony of *Rhizobium leguminosarum bv trifolii* strain WSM1325 was grown in a sterilised nutrient broth and placed on a shaker at 30^{o}C for 48 hrs. Cells were collected by centrifugation at 3000 g for 20 min at 4^{o}C in a Sorvall ™ RC 6 Plus centrifuge (Thermo Scientific). Cells were washed with sterile deionised water and re-suspended in sterile deionised water to a final concentration of 10^8 CFU ml^{-1} measured with a M501 Single Beam Scanning UV/ visible spectrophotometer at 600 nm. In this study, a seed inoculation method was used to introduce the PGPB. Prior to inoculation, seeds of *Brassica juncea (L.) Czern* were surface sterilized with 5 % NaClO for 15 min and washed three times with sterile deionised water. The sterilised seeds were soaked for 4 hrs in 10 mL bacteria suspension (inoculated pots) and 10 mL of sterilized deionized water (control pots) before sowing 5 seeds into each pot containing 600 mg Zn kg^{-1} ($ZnSO_4$, and ZnS nanoparticles). Pots were individually watered (tap water) two times per week to maintain the soil moisture during plant growth. Leachates from each pot were collected from the saucers and returned to the soil. Emergent seedlings were thinned out to 3 plants per pot at 12 days after planting. The pot experiment was conducted in a greenhouse set to provide a day/night temperature of 21 °C in an 18 h photoperiod at a photosynthetic photon flux density (PPFD) of 150 µmol m^{-2} s^{-1} provided by cool white fluorescent bulbs. Plant growth parameters were measured at different growth stages starting from one week after planting. Plant height was measured once a week on each replicate and for each treatment. Toxicity was evaluated by monitoring plant height, and making other observations such as leaf chlorosis,

necrosis, and senescence, during the experiment and determining root length and dry biomass at the end of the experiment.

Table 7.1 Description of experimental treatments

Codes	Treatments
Control	Control without Zn treatment
$ZnSO_4$	*B. juncea* grown on $ZnSO_4$ soil
ZnS nanoparticles	*B. juncea* grown on ZnS nanoparticles soil
B Control	*R. leguminosarum* control without Zn treatment
B $ZnSO_4$	*R. leguminosarum* + *B. juncea* grown on $ZnSO_4$ soil
B ZnS nanoparticles	*R. leguminosarum* + *B. juncea* grown on ZnS nanoparticles soil

7.2.3 Plant harvest, rhizosphere and bulk soil sampling

All plants were harvested 6 weeks after planting. Shoots were separated from roots using scissors, and placed into labelled paper bags. Roots were shaken to remove loosely adhering soils, which was classified as rhizosphere soils. Bulk soil was collected outside the rhizosphere. Each rhizosphere and bulk soil samples represented a mixture of the soil from 3 replicate pots for each treatment. Two duplicate subsamples of the mixed soil were analysed. Roots were washed gently with tap water and root length measured. All samples were transferred to polyethylene bags and taken to the laboratory. Dry plant samples were weighed and ground (mortar and pestle) and two duplicates subsamples of the mixed plant materials were analysed for total metal content using digestion method as described in Chapter 3 (Allen et al., 1974). All plant materials and soil samples were analysed for total Zn using inductively coupled plasma – optical emission spectroscopy (Perkin Elmer Optima 5300 DV ICP-OES). Blanks from both digests were deducted from the analytical result. Both results were reported as the mean of two sub samples of each material. The bulk and rhizosphere soil sample from

each pot was also homogenised separately for pH determination as described in Chapter 3.

7.2.4 X-ray absorption spectroscopy (XAS) studies on soil and plant roots

Changes in Zn speciation between bulk soil, rhizosphere soil and plant roots of *Brassica juncea* grown in 600 mg Zn kg^{-1} (with and without plant growth promoting bacteria) were examined on Beamline B18 at the Diamond Light Source, Didcot, UK. Duplicate samples from roots, bulk and rhizosphere soil were freeze - dried prior to XAS analysis. Bulk and rhizosphere soils were finely ground using a pestle and mortar and ~ 40 mg of each soil sample was mixed with 150 mg cellulose and pressed into pellets of ~ 1mm thickness under a pressure of 2 tonnes. Freeze- dried plant roots were also finely ground. Root samples were placed on a sapphire disc, covered with kapton tape and loaded into a Perspex sample holder, inclined at an angle of 45^{o} to the incident beam which was cryofixed with liquid nitrogen. Beamline B18 was operating in qexafs mode setup with a fast scanning Si (111) double crystal monochromator which was calibrated using Zn foil. Data was collected in fluorescence mode using a 36 -element Ge solid state detector. On average 10 scans (5 mins) were acquired to improve the signal: noise ratio of the data. XAS Zn standards were also finely ground and ~ 15 mg mixed with 150 mg cellulose into pellets. XAS data for Zn citrate, Zn carbonate, Zn acetate and Zn sulfate standards were collected in transmission mode with the ion chamber filled with inert gas to optimize sensitivity. XAS data for the remaining Zn model compounds were collected in fluorescence mode. Data were also recorded simultaneously from a reference Zn foil with a third ion chamber. See Chapter 3 for detailed description of the synthesis of zinc reference compounds.

7.2.5 Data processing and linear combination modelling

An XAS spectrum represents a combination of all Zn species present in the sample transected by the beam (Monsant et al., 2011). XANES spectra were energy normalised using the reference energy of Zn foil and replicates of each sample spectra were merged. In order to assess chemical species information Linear

Combination Fitting (LCF) was carried out in Athena using IFFEFIT software (Ravel and Newville, 2005) to identify the relative proportions of reference compounds within the data of root, bulk and rhizosphere soil samples. The resulting Zn speciation in each sample is dependent on the choice of reference spectra available to fit the sample spectra. The residual value (R) was used to evaluate the reliability of the fitting for each sample, the combination of standards with the lowest r value was chosen as the likely set of components (Gaur et al., 2012).

7.3 Statistical analysis

All treatments were replicated three times and the means (per pot) and standard error (SE) of plant height, and root length were calculated in Microsoft Excel 2013. All treatment means were normally distributed ($p > 0.05$) using Anderson-Darling's normality test. The comparison of treatment means of plant height, root and shoot biomass was conducted by one-way analysis of variance (ANOVA) using Minitab software version 17 (Minitab ™ Inc., USA). If a significant difference is observed between treatments, Tukey's test was used for multiple comparisons with a significance level of $p < 0.05$. A two-way ANOVA was conducted on the overall effects of Zn species and inoculation on plant growth parameters using Minitab software version 16 (Minitab ™ Inc., USA).

7.4 Results

7.4.1 Effect of Zn on plant growth

Observations of plant growth and biomass confirmed the results from previous chapters (5 and 6) comparing $ZnSO_4$ and ZnS nanoparticles as well as the relative effects of *Rhizobium leguminosarum*. The effect of Zn on plant growth and health was monitored from the week of planting for 6 weeks until harvest. All plants appeared healthy during growth but, after 4 weeks of exposure to Zn, the un-inoculated $ZnSO_4$ treated plants appeared paler, had stunted growth and yellowing of leaves compared to plants in the control and ZnS nanoparticles treatments.

Plant height was measured from the base of plant to the tip of the three replicates seedlings in each pot after 6 weeks. Relative to the uninoculated control, *Brassica juncea* plant height was significantly lower in the $ZnSO_4$ treatments ($p < 0.05$) compared to controls after 6 weeks of growth (Figure 7.1a). In inoculated $ZnSO_4$ and ZnS nanoparticles treatments, plant height was significantly higher compared with plants grown in uninoculated $ZnSO_4$ and ZnS nanoparticle soil treatments (Figure 7.1a). Plant roots, which are in direct contact with the Zn in soil, showed a similar trend between treatments. The maximum mean root length after 6 weeks growth was measured in the inoculated control (22 cm) and was significantly higher compared to root length in the uninoculated $ZnSO_4$ and ZnS nanoparticle treatments (Figure 7.1b). However, inoculated plants grown in $ZnSO_4$ and ZnS nanoparticles soil had longer roots after 6 weeks of growth compared to uninoculated plants in the Zn treatments (Figure 7.1b and Figure 7.2). Overall, there are significant effects of Zn treatments and inoculation on plant height and root length ($p<0.05$).

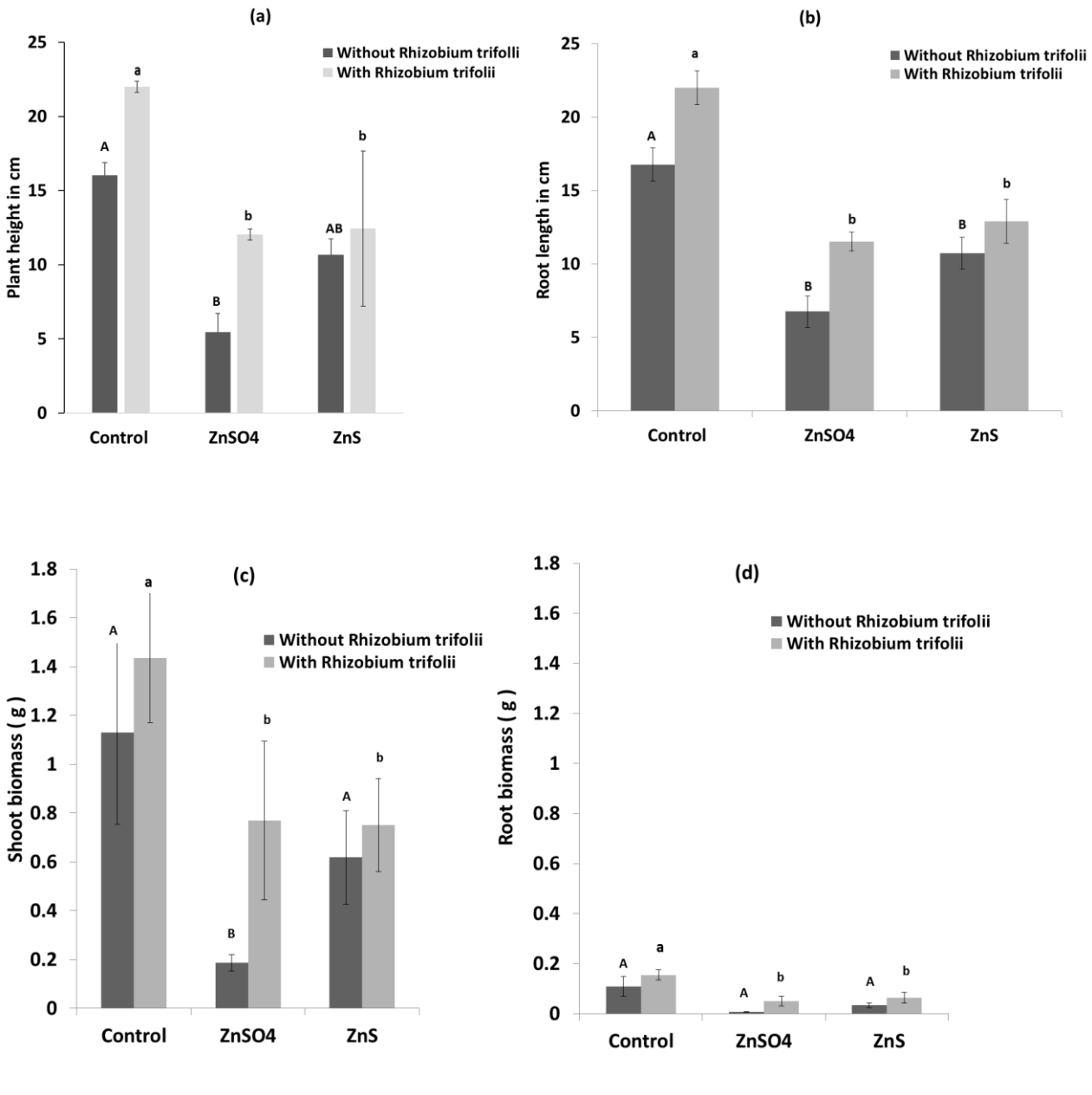


Figure 7.1: Effect of Zn treatments (600 mg Zn kg^{-1}) on uninoculated and inoculated *Brassica juncea* after 6 weeks of growth. (a) Plant height, (b) root length and (c) shoot biomass (d) root biomass. Bars are means and standard error of means of three pots. Significant differences are indicated by different letters at $p < 0.05$ following Tukey multiple comparison tests

Figure 7.2: Photographic illustration of inoculated and uninoculated Brassica juncea (L.) Czern plants in control and Zn contaminated soil (600 mg Zn kg^{-1}) 6 weeks after planting (A) uninoculated treatments and (B) inoculated treatments

Mean shoot and root dry biomass was higher in the inoculated Zn treatments ($ZnSO_4$ and ZnS nanoparticles) compared to the uninoculated treatments. Thus, there is significant effect of Zn treatments and inoculation on shoot and root biomass ($p<0.05$).

The addition of $ZnSO_4$ to soil was shown to affect plant height and shoot biomass more than in ZnS nanoparticles treatment. Based on 600 mg Zn kg^{-1} addition, $ZnSO_4$ treatment is more toxic to *B. juncea* than ZnS nanoparticles. However, inoculated plants had greater plant height, root length and biomass for both Zn treatments indicating that *Rhizobium Leguminosarum (bv) trifolii* compensated for the negative effect of Zn on plant growth.

7.4.2 Zn concentration in plant biomass and soil

The Zn concentration measured in shoots, roots, and soil of *Brassica juncea* in the different experimental treatments are presented in Figure 7.3. Zn concentrations in the shoots were much higher than in the respective plant roots for all Zn treatments (Figure 7.3a).

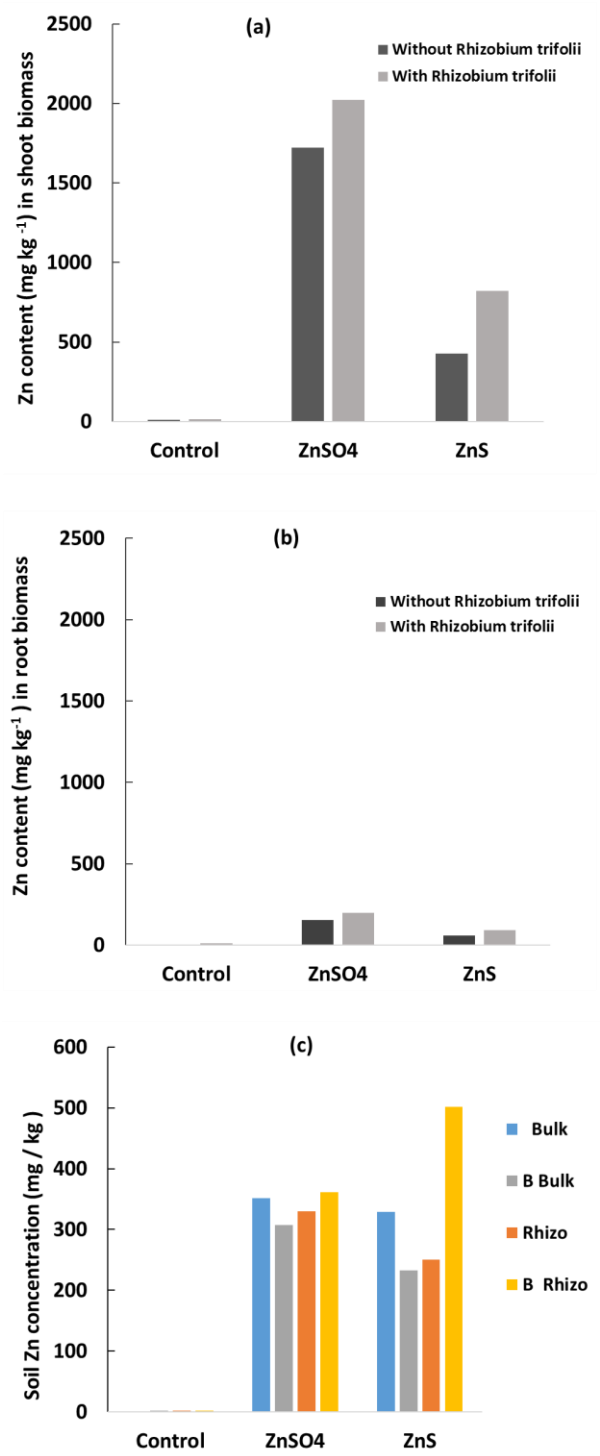


Figure 7.3: Zn concentrations in (a) inoculated and uninoculated shoot biomass, (b), inoculated and uninoculated root biomass (c) inoculated and uninoculated bulk and rhizosphere soil at 6 weeks after planting in Zn contaminated soil. Values are means of two sub samples of each treatment.

The maximum Zn concentrations in shoots and roots of *Brassica juncea* was observed in the $ZnSO_4$ treatment, indicating more Zn mobilization to plants treated with $ZnSO_4$ than in ZnS nanoparticles. Nevertheless, Zn concentrations in shoots and roots were more in both Zn treatments compared to the control. Inoculation with *Rhizobium leguminosarum (bv) trifolii* greatly enhanced the quantity of Zn in shoot tissues compared with the respective non-inoculated controls. A similar effect was observed in root Zn concentration in plants inoculated with *Rhizobium leguminosarum (bv) trifolii* compared to the uninoculated plants for all Zn treatments.

The concentration of Zn in inoculated and uninoculated bulk and rhizosphere soils was analysed separately following plant harvest (Figure 7.3c). Soils contaminated with $ZnSO_4$ and ZnS nanoparticles contained different concentrations of Zn after 6 weeks of growth. Zn concentrations in inoculated and uninoculated ($ZnSO_4$ and ZnS) bulk and rhizosphere soils were lower than the initial Zn content (600 mg Zn kg^{-1}) (Figure 7.3c), a likely consequence of Zn uptake by *Brassica juncea.* For both Zn treatments, the uninoculated rhizosphere soil contained lower Zn contents than in the corresponding bulk soil. In the inoculated Zn treatments, Zn concentrations increased in the rhizosphere soils and decreased in the bulk soil, compared to the uninoculated Zn treatments.

To investigate a possible mechanism by which bacteria mobilise Zn from the bulk soil, pH of rhizosphere and bulk soils was measured after plant harvest. The rhizosphere soil pH varied between 6.42 and 7.63, and the bulk soil pH varied between 6.90 and 7.75 (Table 7.2). The rhizosphere soils are more acidic than the bulk soils with differences in the range of 0.1-0.4 pH units. From the initial soil pH of 6.2 the inoculated and uninoculated $ZnSO_4$ treatments had lower pH compared to ZnS nanoparticle-treated soils.

Table 7.2: pH of the rhizosphere and bulk soils after growth of Brassica juncea (L.) Czern. pH values are means of two sub samples of each treatment.

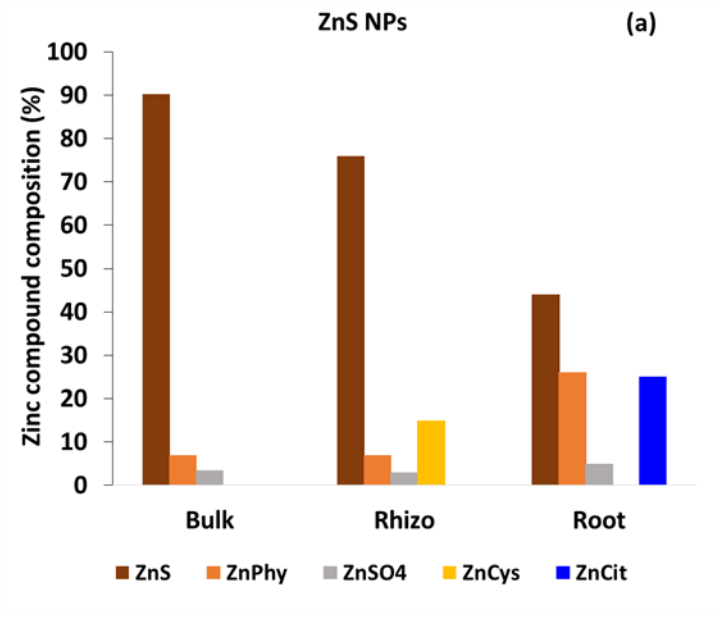
Soil initial pH 6.2

Treatment	**Bulk soil**	**Rhizosphere soil**
Control	7.75	7.63
B Control	7.55	7.46
$ZnSO_4$	6.90	6.71
$BZnSO_4$	6.81	6.42
ZnS	7.64	7.52
BZnS	7.61	7.41

7.4.3 µ-XAS analysis of Zn speciation and distribution in roots, bulk and rhizosphere soil

µ-XAS was employed to investigate the speciation of Zn in the bulk soil, the rhizosphere soil and in the roots of *B. juncea* treated with 600 mg kg $^{-1}$ Zn ($ZnSO_4$ and ZnS nanoparticles 6 weeks after planting. To obtain quantitative information on Zn speciation, XANES spectra were interpreted using linear combination fitting using spectra from selected Zn standards listed in 7.2.5. The LCFs with the lowest R factor for roots, bulk and rhizosphere soil treated with ZnS nanoparticles and $ZnSO_4$ including the Zn compound composition (%) are shown in Figures 7.4 for ZnS NPs, and Figures 7.5 for $ZnSO_4$ treatments.

The LCF result confirms that Zn in uninoculated plant roots exposed to ZnS NPs was predominantly in the form of ZnS (44 %), the rest being in the form of Zn phytate (26 %), Zn citrate (25 %) and Zn sulphate (5 %) (Figure 7.4a). Zn forms in the inoculated ZnS NPs plant root were slightly different to those of the uninoculated, Zn forms was predominantly in the form of Zn phytate (33 %), the rest being in the form of Zn cysteine (30 %), Zn citrate (30 %), and Zn formate (7 %) (Figure 7.4b).



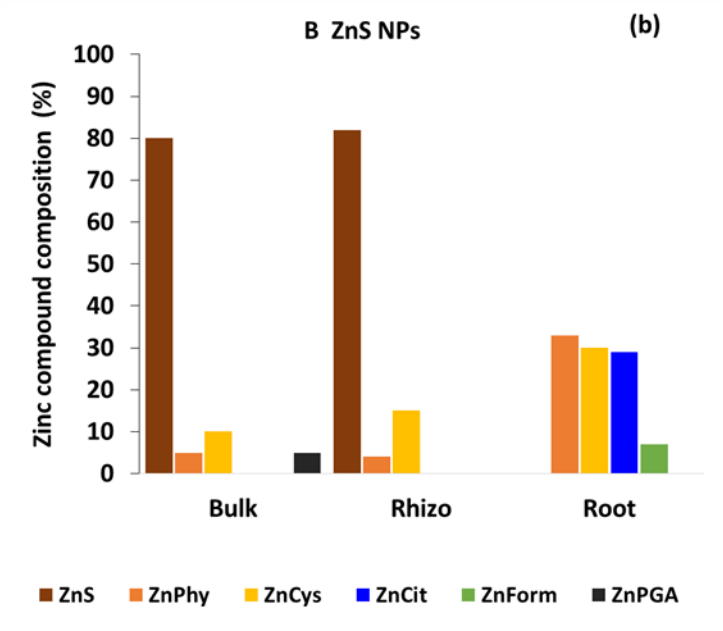


Figure 7.4: Zinc compound composition (%) in (a) uninoculated, (b) inoculated bulk, rhizosphere soil and roots, of ZnS NPs treatment. ZnS- ZnS NPs, ZnPhy- Zn phytate, $ZnSO_4$- Zn sulphate, ZnCys- Zn cysteine, ZnCit- Zn citrate, ZnForm- Zn formate, ZnPGA- Zn polygalacturunic acid.

The LCF of rhizosphere and bulk soil from the ZnS nanoparticles treatment were compared to see whether there are differences in Zn speciation. For the

uninoculated bulk soil, 90 % of the total Zn was in the form of ZnS with the remaining Zn identified as phytate (7%) and sulphate (3%) (Figure 7.4a). In the inoculated bulk soil, about 80 % of the Zn was present as ZnS with the remaining Zn associated with cysteine (10 %), PGA (5 %) and phytate (4%) (Figure 7.4b).

In the uninoculated rhizosphere soil 76 % of Zn was in the form of ZnS, with the rest occurring as Zn cysteine (15%), Zn phytate (2%) and Zn sulphate (2%) (Figure 7.4a). In the inoculated rhizosphere soil, Zn was mainly in the form of ZnS (82 %), with Zn cysteine (15 %) and Zn phytate (4%) (Figure 7.4b). This shows that more Zn occurred as ZnS in the soil but less in roots indicating transformation of Zn to other compounds in the roots.

Although, the LCF fits for $ZnSO_4$ treatments were not as good as for the ZnS nanoparticles treatment (higher R values), they showed similar features for rhizosphere, bulk soil samples and the roots. The LCF of inoculated and uninoculated roots of *Brassica juncea* grown in soil treated with $ZnSO_4$ showed different speciation of Zn from those grown in the ZnS nanoparticles treatment (Figure 7.5).

The best LCF fits for uninoculated roots in the $ZnSO_4$ treatments showed Zn was in the form of histidine (38 %) > carbonate (24%), citrate (18 %) > phytate (16%) (Figure 7.5a) while for the inoculated roots Zn was in the form of histidine 43%, cysteine (31%), formate (16%) and oxalate (7%) (Figure 7.5b).

The LCF for bulk and rhizosphere soil had the same trend of Zn forms in both inoculated and uninoculated treatments, with Zn predominantly in the forms; carbonate> sulphate >histidine> phytate in different percentages (Figure 7.5 a –b).

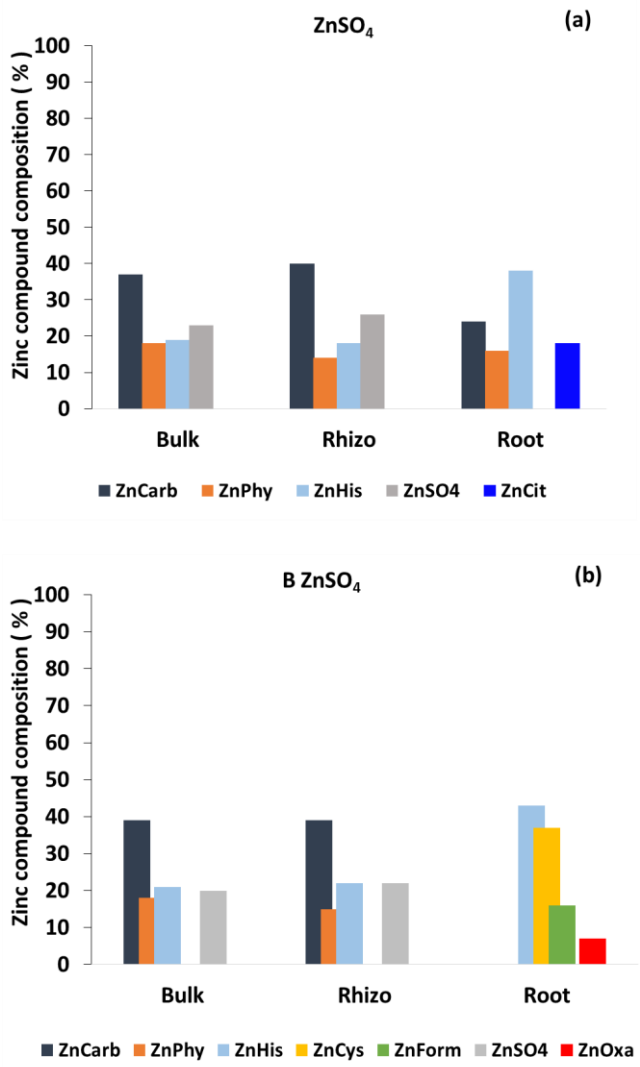


Figure 7.5: Zinc compound composition (%) in (a) uninoculated (b) inoculated bulk, rhizosphere soil and roots of $ZnSO_4$ treatment. ZnCarb- Zn carbonate, ZnPhy- Zn phytate, ZnHis- Zn histidine, $ZnSO_4$- Zn sulphate, ZnCys- Zn cysteine, ZnCit- Zn citrate, ZnForm- Zn formate, ZnOxa- Zn oxalate.

Overall, not much changes in speciation between bulk and rhizosphere soil but there was a significant change in the proportions of the different species between the rhizosphere and roots. Thus, these differences in Zn speciation in roots and rhizosphere soil are dependent on the form of Zn contamination.

7.5 Discussion

7.5.1 Zn speciation changes across the soil-rhizosphere-plant interface

The plant- soil interface has different physical, biological and chemical characteristics from the bulk soil due to strong influences of plant roots (Seshadri et al., 2015). Identifying Zn speciation changes across the soil-rhizosphere-plant interface in *Brassica juncea* is crucial for elucidating the mechanisms by which plant growth promoting bacteria ameliorate toxicity in Zn hyperaccumulator plant species growing on Zn contaminated soil. Specifically, to *Brassica juncea* investigated in this study, we confirmed (1) the existence of speciation gradients between the rhizosphere and plant roots and (2) that *R. leguminosarum* modified Zn speciation across the rhizosphere and plant root depending on the form in which Zn was spiked in soil.

Zinc is acquired from the soil primarily as Zn^{2+} but also potentially complexed with inorganic and organic ligands, and transport to the shoot through the xylem (Broadley, et al., 2007; Alloway, 2008). Comparison of the XANES results for bulk and rhizosphere soils, showed no differences in the speciation of Zn between inoculated and un-inoculated treatments when Zn was supplied in the form of $ZnSO_4$ (Figure 7.5). In both treatments for $ZnSO_4$, speciation was dominated by Zn carbonate with almost equal proportions of Zn sulfate and Zn histidine, but slightly lower Zn phytate. A steep increase in the proportion of Zn histidine between the rhizosphere and the root tissue was also observed. Histidine is an essential amino acid with a positively charged imidazole functional group (Chikrabarti, 1990, Gluster, 1991; Gramlich et al., 2013). The occurrence of Zn-His in roots in this present study agrees with previous studies of Zn hyperaccumulator species (Lasat et al., 1998; Salt et al., 1999). Complexation with a high affinity compound such as Zn-histidine would assist non-toxic Zn mobilization thus protecting plants from deleterious effects (Yadev, 2010). A significant effect of bacteria inoculation in the $ZnSO_4$ is the appearance of Zn cysteine and small amounts of Zn formate and Zn oxalate in the root tissue (Figure 7.5b), accompanied by the disappearance of Zn carbonate and Zn phytate. Apparently, the decrease of Zn carbonate in roots of uninoculated plants

was due in part to the presence of Zn citrate, which was not detected in the soil. Neither inoculated nor uninoculated $ZnSO_4$ treatment results in the presence of Zn sulfate in root tissue. This observation is in contrast to the findings of Adediran et al. (2015) in which a significant amount of Zn sulfate was inferred in the absence of bacteria, although the author did report a relatively poor linear combination fitting of the data, and also used a different soil formulation (soil with higher organic content). Significantly, this study confirms the importance of Zn cysteine complexation in roots of bacteria inoculated plants as reported previously (Adediran et al., 2015; 2016), and further that the transformation occurs within the plant (epidermal) tissue (Adediran et al., 2016). The absence of ZnS in the $ZnSO_4$ system indicates that the soil does not attain sufficiently reducing conditions to induce sulfate reduction in the 6 week growth period, although occurrence of reducing condition is highly unlikely, given the low organic matter content of the soil used, even if sulfate reducing bacteria were present. Thus, there is a transition in speciation between the rhizosphere and plant roots in the form of cysteine when bacteria are present. More generally, there is an increase in Zn histidine between rhizosphere and roots, whereas sulfate disappears across this interface.

Linear combination fitting of the acquired XANES data confirmed that in the ZnS NPs system, Zn speciation is dominated by ZnS in both uninoculated and inoculated treatments (except in inoculated roots) (Figure 7.4 a-b). However, there is a transition in speciation between the rhizosphere and plant roots when bacteria are present (Figure 7.4b). The transition from rhizosphere to plant tissue is characterised by a steep drop in the proportion of ZnS and the absence of Zn cysteine, accompanied by a steep increase in Zn phytate and Zn citrate in the uninoculated ZnS treatment. The only major difference between inoculated and uninoculated ZnS treatments is the total absence of ZnS in the inoculated root which was replaced by Zn cysteine and Zn formate and also the occurrence of Zn cysteine in the bulk soil of the inoculated treatment (Figure 7.4 a-b). Small amounts of Zn sulfate were inferred across all three compartments in the uninoculated treatment but it was absent in the inoculated treatment. Zn cysteine appears across the rhizosphere-root transition concomitant with a rise in Zn phytate and Zn citrate.

It also appears that *R. leguminosarum* induced transformation of Zn to formate in inoculated roots.

Comparison of $ZnSO_4$ and ZnS treatments shows that overall ZnS treatments led to more formation of Zn cysteine in soil and roots compare to $ZnSO_4$ treatments. The formation of cysteine in roots of plants is closely linked to sulfate metabolism, in which sulfate is first converted to sulphide, which combines with O-acetyl serine to form cysteine (Leustek and Saito 1999; Leustek, 2002; Adediran et al., 2015). Although cysteine was present in ZnS treated soil, the absence of Zn cysteine complexes in uninoculated roots treated with ZnS (Figure 7.4a) may imply insufficient sulfate to trigger formation of cysteine in roots. The solubility product constant of ZnS is $\sim 10^{-23}$ $(mol/L)^2$ (Clever et al., 1992) at the circum-neutral pH measured in the bulk and rhizosphere soil. In principle, the production of root exudates should acidify the rhizosphere and help to solubilize Zn (Dessureault-Rompré et al., 2008) but the measured pH changes in our soils were small (~0.3) (Table 7.3). These small changes also imply oxidative dissolution (which can promote faster acidification) was minimal, although the presence of Zn sulfate species in the uninoculated ZnS treatment does testify to its occurrence (Figure 7.4a). The possibility of oxidative dissolution of ZnS by plants has been reported, but it is time dependent (Panfill et al., 2005; Voegellin et al., 2011) and although none of these studies examined speciation changes across soil-rhizosphere-plant interfaces. On the contrary, the presence of Zn cysteine in inoculated soils and roots exposed to ZnS may suggest a different route to cysteine synthesis in these treatments. Rhizobium bacteria are known to supply their own cysteine requirements in the absence of supplies from symbiotic plants, through direct synthesis from sulphide and serine (Kredich and Tomkins, 1996). Plants contain two major types of cysteine: (1) rich low molecular weight metal binding peptides, the metallothioneins (MTs) and (2) the phytochelatins (made up of three amino residues gly-cys-glu) which are both assumed to be involved in accumulation, detoxification and metabolism of metal ions including Zn (Grill et al., 1985). This might also account for the presence of cysteine in inoculated roots treated with $ZnSO_4$, showing better zinc tolerance to *Brassica juncea*.

Another difference between $ZnSO_4$ and ZnS nanoparticles is the appearance of Zn phytate in the inoculated roots exposed to ZnS nanoparticles. Zn released in ZnS NPs plant root must have been immobilized as Zn phytate. Phytate myoinositol hexakisphospahte contains a negative charged phosphate group but forms stable complexes with ions including Zn (Raboy et al., 2001; Lv et al., 2015). Although, with Zn it is precipitated from solution due to its less soluble form (Champagne and Fisher, 1990). The formation of Zn phytate are known processes for Zn immobilization in roots (Van Steveninck et al., 1994). Moreover, several investigations have reported that the accumulation of Zn-phytate would be an effective rapid response against the transport of excessive Zn to the shoot in plants (Van Steveninck et al., 1994; Terzano et al., 2008; Kopittke et al., 2011). Recently, Adediran et al. (2015) reported that *R. leguminosarum and P. brassicacerum* improved plant growth and increased Zn accumulation in roots of *Brassica juncea* due to the presence of Zn phytate.

The most prominent similarity between the $ZnSO_4$ and ZnS NPs treatments is the presence of Zn formate in roots of plant grown from seeds inoculated with *Rhizobium leguminosarum*. Inoculation with *Rhizobium leguminosarum* promoted the formation of Zn formate in both ZnS and $ZnSO_4$ treatments which is also a potential ligand involved in plants during tolerance and detoxification of toxic metals (Rauser, 1995; Kumar et al., 2012).

Lastly, Zn citrate is a prominent species in roots from ZnS-spiked samples and was also identified in roots in the uninoculated $ZnSO_4$ treatments. It is not present in any of the soils, suggesting that citrate ligands are supplied by the roots in the form of exudates, consistent with previous studies (Dessureault-Rompré et al., 2008), although other studies rule out organic complexation of Zn in the root tissue (Medas et al., 2015).

Zn coordination can occur with both organic and inorganic Zn ligands in the rhizosphere (Gardea-Torresdey et al., 2005; Terzano et al., 2006). Zn coordination

with high affinity organic and inorganic ligands in the rhizosphere including its interaction with PGPB, may be a mechanism in reducing phytotoxicity caused by Zn^{+2} and promoting plant adaption on Zn contaminated soil. The estimate of Zn speciation by XAS across the soil-rhizosphere-plant interface is dependent on the best fit of the spectra of the selected Zn standards to the sample spectra of the different Zn treatments. Consequently, results from the LCF analysis are indicative and not absolute (Lombi and Susini, 2009; Monsant et al., 2011).

7.5.2 Effects of Zn and *R. leguminosarum* on plant growth and uptake

The growth of *Brassica juncea* in this study differed in response to the different Zn treatments. Exposure to $ZnSO_4$ inhibited growth of *Brassica juncea* more than ZnS nanoparticles exposure (Figure 7.1). Other observed symptoms of toxicity, such as stunted growth and yellowing of leaves, were exhibited in plants grown in soil contaminated with $ZnSO_4$ but not with ZnS nanoparticles. The Zn concentration used in this study (600 mg Zn kg^{-1}) may account for reduction in plant growth and biomass production through impairing plant metabolism and interfering with the absorption of essential elements (Duman and Ozturk, 2010). *Rhizobium leguminosarum* is a known rhizosphere bacterium that resides and colonises the soil root interface (Glick, 1995; Reeve et al., 2010; Adediran et al., 2015; 2016) eliciting growth promotion in plants. Inoculated plants showed strong differences in all plant growth parameters compared to uninoculated plants with inoculation improving plant growth in Zn contaminated soil (Figure 7.1). This suggest that interactions between bacteria and roots in the rhizosphere are responsible for stimulating plant growth. Although, not measured here, other studies suggest that the most likely mechanisms are through the synthesis of phytohormones, (Gupta et al., 2002), indole acetic acid (IAA) (Glick et al., 1998) and ACC deaminase activity which alters plant metabolism resulting in healthier plants (Sheng et al., 2008; Croes et al., 2013; Ma et al., 2015; Adediran et al., 2015). This is the most contributed factor which may explain these strong differences between the growth of un-inoculated and inoculated plants, since plants have evolved along with rhizosphere microbiome that contributes to the growth and health of the plant (Pieterse et al.,

2014). Plant growth promoting bacteria alone and in combination with their plant host influence metal availability in soil (Verme et al., 2010).

Rhizobium leguminosarum significantly enhanced Zn accumulation in plants grown in soil amended with $ZnSO_4$ than with ZnS nanoparticles (Figure 7.3). However, there was higher Zn concentration in the shoots of the inoculated ZnS nanoparticles treatment compared to the uninoculated treatment. PGPB are able to solubilize unavailable forms of metal by producing siderophores and other metal-chelating substances (Verme et al., 2010). The solubilisation of ZnS by PGPB is dependent on the potential to oxidize sulphide ions (Fowler and Crundwell, 1998). It is likely that the Zn concentration in shoot (Figure 7.3) from ZnS nanoparticles treated soil may be as a result of slow Zn^{2+} released from ZnS nanoparticles treatment.

The lower soil Zn content after plant harvest showed inoculated plants were most effective at decreasing the total Zn from the bulk soil by migrating more Zn into the rhizosphere through the roots for uptake by *B. juncea* (Figure 7.3c). This confirms previous findings that PGPB can stimulate metal uptake by plants (Chen et al., 2013; Adediran et al., 2015). The mobility of Zn in plants is dependent on its availability in soil (Broadley et al., 2007; Hafeez et al., 2013). When Zn is bound in reduced S complexes it becomes unavailable to plants as it is not mobile, hence in the ZnS nanoparticles treatment more ZnS remained in the soil than was taken up by plants. Consequently, plants grown in the ZnS nanoparticles treatment soil acquired less Zn from the soil, reducing Zn toxicity effects on plants. This result is in agreement with the observation of Whiting et al. (2001 a) who reported less Zn accumulation in a Zn hyperaccumulating species grown on ZnS amended soil than in soils treated with other Zn forms (Zn sulphate, phosphate and Zn oxide).

7.5.3 Root induced changes in pH and effects on Zn mobility and uptake

Soil pH has a dominant effect on solubility, availability and phytotoxicity of ions (Clark and Baligar, 2000), which governs the speciation of Zn in soil (Alloway, 1995).

The soil-root interface is characterized by high concentrations of easily degradable compounds exuded from the roots (Vranova et al., 2013), especially at the root tip (Kaiser at al., 2015). The excretion of protons and exudation of organic compounds such as organic acids, released by plant roots or microbes, may play a role in increasing the acidity of the rhizosphere environment (Nye, 1981; Strom et al., 2002; Hinsinger et al., 2009) compared to bulk soil. An increased acidity (low pH) in the rhizosphere will also increase metal solubility and eventually phytoextraction potential (Li and Wong, 2010). The optimal soil pH for plant growth has been documented to be around 6.4 and for some soils from 6.0-7.2 (Putnams et al., 1990). For a typical *Brassica* species optimal soil pH for growth has been reported to be 6.5 (Zaurov et al., 1999). The soil used in this study was mildly acidic, but rhizosphere pH was lower compared to the bulk soil (Table 7.2) in measurements conducted after plant harvest which may have been influenced by the high microbial activity in the rhizosphere. The soil in the $ZnSO_4$ treatment had a lower pH than in the ZnS nanoparticles treatment probably due to increased mobile Zn^{2+} ions displacing H^+ ion bound electrostatically to the negative surfaces of the soil (Alloway, 1995). Since ZnS are observed to be insoluble in soil environments (Whiting et al., 2001; Uena et al., 2004; Voegelin et al., 2011), increased soil pH, especially above 6.5 as recorded in this experiment in both the rhizosphere and bulk soil of the ZnS nanoparticles treatment will lead to formation of Zn phosphate (Yang et al., 2011) which is potentially less toxic to plants. Differences in the mobility of Zn in the bulk and rhizosphere of *Brassica juncea* may be attributed to changes in pH. However, increased Zn concentration in *B. juncea* associated with Zn supply, indicates that Zn uptake by *Brassica juncea* is less dependent upon Zn solubility caused by soil acidification, but rather more dependent on the presence of the PGPB inoculant. Whiting et al. (2001b) reported that increases in solubility of Zn in the soil was not due to changes in pH, but rather PGPB facilitated increases in the solubility of Zn in soil thus enhancing Zn accumulation in *Thlaspi caerulescens.* Therefore, in this present study, root associated microorganisms including *Rhizobium leguminosarum* in the rhizosphere provided a positive link between roots and the soil, thus leading to a lower pH in the rhizosphere soils and increasing Zn concentration and accumulation in *Brassica juncea*. *R. leguminosarum* as a PGPB may affect the soil-

plant interface system by mobilizing Zn by different mechanisms through chelate formation, siderophore production, rhizosphere acidification (Marschner, 1995; Mandal et al., 2007) thereby facilitating Zn phytoextraction process.

7.6 Conclusion

X-ray synchrotron-based techniques and data analysis were suitable for investigating and quantifying Zn forms in the plant- root-interphase. The rhizosphere of Zn accumulating plants is a complex system that provides a niche for adapted Zn resistant *Rhizobium leguminosarum bv. trifolii* critical for understanding phytoremediation. Differences exist in Zn speciation between $ZnSO_4$ and ZnS nanoparticles treatments in plant- root-interphase indicating different uptake mechanisms of Zn in *Brassica juncea (L.) Czern.* Our study established the existence of a speciation gradient between the plant roots and rhizosphere. However, speciation gradients were not only associated with inoculation with PGPB but also with the form of Zn in the soil. PGPB modified speciation changes across the rhizosphere –root interface based on the form in which Zn was spiked in soils, with ZnS favouring the formation of Zn cysteine, and Zn formate while $ZnSO_4$ led to the formation of Zn cysteine in plant roots. Thus this study clearly indicates that Zn speciation is a strong factor influencing the plant -root interphase, rather than the total Zn concentration in soil.

Chapter 8

8 General discussion

Metal contamination including metal nanoparticles is ubiquitous in the environment and is of increasing environmental concern since metal persistence in the environment is indefinite (Ding et al., 2010). Zinc is essential for plants as plants absorb Zn as a divalent cation (Zn^{2+}) which acts as a regulatory cofactor of a larger number of enzymes (Prassad et al., 2012). The effect of metal contaminants on entire ecosystems is a function of their bioavailability, solubility and mobility (Scheckel et al., 2009). These effects are often not dependent on the total concentration of metals in soil, which is not sufficient for estimating the risk of metals in the soil environment but rather the speciation of metals, as speciation or chemical form of metal governs its fate, toxicity, mobility and bioavailability in soil environment (Adamu et al., 2013). Phytoextraction utilizes hyperacumulating plants to extract metals and concentrate them in aboveground biomass (Chaney et al., 1997; Zhang et al., 2014). Moreover, the addition of plant growth promoting bacteria to hyperaccumulator species used in phytoremediation practices makes the remediation process more effective (Gamalero and Glick, 2012; Glick, 2010).

This study investigated the effect of metal speciation on Zn plant dynamics in the presence of plant growth promoting bacteria. The major findings from this work are:

1. Zn uptake, growth and toxicity in *Brassica juncea* was dependent on the forms in which Zn was exposed in soil.

2. *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* had a growth promoting effect on plant biomass production in Zn contaminated soil and the use of these PGPB was more effective in alleviating Zn toxicity in $ZnSO_4$ treatments than in Zn nanoparticles treatments.

3. The presence of bacteria changes speciation that in turn promoted phytoextraction.

In the following sections, I expand on these findings and use them to construct a conceptual model linking Zn speciation and PGPB on Zn uptake and toxicity by *Brassica juncea*.

8.1 Summary of findings

8.1.1 Zn resistant PGPB and seed germination

A preliminary study on the toxicity of Zn to PGPB was initially conducted to examine the effects of Zn on the growth of *Rhizobium leguminosarum* and *pseudomonas brassicacaerum* using nutrient broth and Hoagland solution with different concentrations of Zn (100, 200, 400, 600 and 800 mg L^{-1}) using $ZnSO_4$, as well as ZnO and ZnS nanoparticles (Chapter 4). The dose response experiment revealed a marked difference in bacteria growth between $ZnSO_4$ and nanoparticulate ZnO and ZnS. *Rhizobium leguminosarum* and *pseudomonas brassicacearum* decreased in growth upon increasing concentration of Zn treatments from 0 to 54 h as compared with the control. The dose response results of these three Zn contaminants as reflected by the optical density and colony forming units revealed that $ZnSO_4$ was the most toxic since it impaired bacterial growth from the lowest concentrations after 54 hours of bacteria growth. Both PGPB exhibited remarkable resistance to the Zn nanoparticles treatments compared to $ZnSO_4$. My investigation revealed the practical bioremediation potential of both study PGPB since they exhibited excellent adaptation or resistance to Zn. However, the use of Hoagland solution was aborted in this study as the solution never supported bacterial growth and as such, the use of hydroponics for studying Zn phytoextraction was discontinued.

Seed germination is regarded as the most critical stage in the life cycle of plants and serves as the immediate exchange interface with the surrounding medium (Solanki and Dhankhar, 2011). Seeds of *Brassica juncea* were additionally tested to examine the effect of Zn speciation on seedling germination and seedling growth and to investigate the role of *Rhizobium trifolii* and *Pseudomonas brassicacearum* in improving tolerance to Zn species. At the Zn concentration (600 mg Zn kg^{-1}) used in

this study, $ZnSO_4$ significantly inhibited seeds (uninoculated) germination rate compared with the control and Zn nanoparticles treatments. As observed, germination percentage was unaffected for both ZnO and ZnS nanoparticles as there were no significant differences. Thus the ability to germinate depends on the plant sensitivity to the different Zn contaminants (Chapter 4). Root and shoot length was also observed to be different for different Zn species, with root length being the most sensitive to Zn toxicity. As such, the order of toxicity of the study contaminants to growth response of plants is $ZnSO_4$ > ZnO > ZnS. Upon inoculation with PGPB, germination percentages and seedling growth was enhanced for all Zn treatments.

Taking into account the Zn concentrations, we confirmed that seed germination and early seedling growth of *Brassica juncea* was affected differently upon exposure to different Zn treatments. Thus this preliminary test, although considered short- term, was a guide highlighting the importance of Zn speciation on plant toxicity and a need to determine whether the applied Zn concentrations would respond differently in a natural soil.

8.1.2 Effect of Zn speciation on plant growth and phytoextraction

A pot experiment was conducted to assess the effect of Zn on plant growth when elevated Zn concentrations are added to soil in the form of soluble Zn or as nanoparticulate ZnS and ZnO nanoparticles by comparing growth, Zn accumulation and toxicity of a hyperaccumulator species (*Brassica juncea* (L.) Czern). Pot experiments (Chapters 5, 6 and 7) conducted during this study showed a systematic trend on the effects of Zn (600 mg Zn kg^{-1}) on *Brassica juncea*. Results on plant height, number of leaves, root length, plant biomass and chlorophyll content on *Brassica juncea* showed that soluble Zn ($ZnSO_4$) was the most toxic form of Zn compared to nanoparticulate forms. Plants remained healthy and grew better in Zn nanoparticles treatments compared to $ZnSO_4$ treatments (Chapter 5, 6 and 7). It is well known that Zn stimulates *Brassica juncea* growth at low concentration (Singh and Sinha 2004; Alia et al., 1995; Prassad et al., 1999) but at high Zn concentration, Zn toxicity sets in (Adediran et al., 2015). Trace metal bioavailability is critically

dependent on bioassessibility and since soluble zinc is more bioaccessible (Arnold et al., 2007), the observed toxicity trends are entirely consistent with respect to $ZnSO_4$ and the nanoparticulate forms. Zn content in plant biomass was higher in plants grown on $ZnSO_4$ (Chapter 5-7) than in the Zn nanoparticles. The uptake of Zn in plants part from Zn nanoparticle treatments are not only related to surface properties, size, shape particle composition, growth matrix, but also dependent on the plant species (Siddiqui et al., 2015). Bioaccumulation factor (BCF) was a useful parameter to evaluate the potential of the plants to remove metals from soil (Ma et al., 2015). My study showed that the bioaccumulation factor (BCF) for all Zn treatments varied and were greater than 1 for $ZnSO_4$ and ZnO nanoparticles treatments suggesting that *Brassica juncea* is a hyperaccumulator of Zn from Zn contaminated soil. Moreover, phytoextraction efficiency which evaluates Zn removal percentage after 6 weeks of plant growth suggests that *Brassica juncea* were most effective in removing zinc from soil treated with $ZnSO_4$ than in Zn nanoparticles.

8.1.3 Environmental implications of addition of different Zn forms to soil

Zinc (Zn) are naturally present and ubiquitous in soil and are important micronutrient (Fekiacova et al., 2015). Human activities contribute to the input of zinc to soil in different chemical forms and has become a focus of environmental risk assessment studies (Peralta-Videa et al., 2011). Soils are the major sinks for metal and metal nanoparticles released into the environment (Pachapur et al., 2016; Gu et al., 2016) which may show potential adverse effects in the environment, making them target compounds for phytoremediation (Gomes et al., 2016). This would emphasise the importance of risk assessment for Zn and Zn NPs in soils. The high Zn concentration in soil pore water exposed to ZnO NPs soil suggest that they would likely be mobile unless their mobility is reduced by aggregation and adsorption mechanism (Chapter 5). This finding indicate ZnO NPs are unlikely to be persistent in soil and risk assessment in the soil environment would be similar to soluble source of Zn. However, the Zn soil pore water (Chapter 5) do not correlate well with Zn concentrations in plant tissues which showed higher Zn concentrations in plant tissues exposed to $ZnSO_4$ than in Zn NPs treatments (Chapter 5,6, and 7).

Thus our findings demonstrate that the behaviour and bioavailability of Zn NPs, when introduced at 600 mg Zn kg^{-1} concentration, are distinguishable from dissolved Zn, indicating that concentration dependent effects must be taken into consideration in risk assessment.

These finding also indicate that phytotoxicity effects on *Brassica juncea* is related to dissolved Zn released. The difference in uptake of Zn in *Brassica juncea* from soil exposed to different Zn treatments indicates that plants responses in Zn contaminated soil were predominantly dependent on Zn speciation. The available data on Zn availability and phytotoxicity of the different Zn forms is contributory to risk assessment. While the overall risk is low for *Brassica juncea* exposed to Zn NPs soil, however, the risk of uptake from Zn NPs treatments is not worse than the soluble Zn treated soil. Therefore, to evaluate risk, our findings indicates that the risk associated with the presences of ZnS NPs in soil would be less than ZnO NPs and more in soluble Zn treated soil.

8.1.4 Role of plant growth promoting bacteria on plant growth

Metal toxicities lead to total decrease in plant growth (Reichman, 2002; Rascio and Navar-Izzo, 2011), but some bacteria known as plant growth promoting bacteria (PGPBs), have been known to counteract Zn toxicity effects and to improve plant growth (Wani et al., 2008; Adediran 2015; 2016). As a result of the poor growth yield in plants exposed to Zn (Chapter 5-7), biologically assisted phytoremediation was considered to salvage plants from Zn toxicity. A pot experiment investigated the role of *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* in ameliorating Zn toxicity in plants grown in $ZnSO_4$, ZnS and ZnO nanoparticles treated soil. Seed inoculation with these strains demonstrated them to have growth promoting effects on *Brassica juncea* thus increasing phytoextraction efficiency (Chapter 6). Pot experiment demonstrated that the application of *Rhizobiium leguminosarum (bv) trifolii* and *Pseudomonas brassicacearum* (Chapter 6) could enhance plant biomass, and Zn uptake with many of these PGPB effects observed on $ZnSO_4$ than in Zn nanoparticles treatments. Both inoculants ameliorated Zn toxicity in plants which may be due to their plant growth promoting properties such

as IAA, siderophores, ACC deaminase, phosphate solubilisation (Noel et al., 1996; Rajkumar et al., 2008; Ma et al., 2011; Santoyo et al., 2016). *Rhizobium leguminosarum* promoted plant root development (Chapter 7) resulting in very vigorous and extensive root system thus creating an active rhizosphere for Zn uptake. However, PGPB generally elicited non-toxic forms in plants by promoting the formation of Zn cysteine in all Zn treatments which is a potential Zn complex involved in tolerance and detoxification of Zn in all plants. Thus, in the light of the results obtained in this study, the potential use of *Rhizobium leguminosarum* and *Pseudomonas brassicacearum* can be considered as a viable tool to enhance plant growth and reduce negative effects of Zn toxicity in *Brassica juncea*. These strains were individually applied to pots in this study, a combination action of both study strains has been shown to be even more effective in remediating Zn contaminated soil (Adediran et al., 2015).

8.1.5 Localization and Zn speciation in *Brassica juncea*

Plants have developed several mechanisms to resist toxic metals. One of these mechanisms is compartmentalization of metal in specific cellular organelles or tissues (Kupper et al., 1999; Kramer et al., 2000; Isaure et al., 2006; Wojcik et al., 2005). Plant roots are very important as they are main contact through which Zn gains access into the plant. Using TEM the spatial distribution of Zn in roots of *Brassica juncea* treated with 600 mg Zn kg^{-1} showed that Zn was preferentially distributed in the form the plant was exposed to in Zn contaminated soil. Zn was localized in the epidermal cells, cell wall and the endodermis in agreement with µXRF, which may be important for plant defence against Zn toxicity (Chapter 6). Although sensitivity of TEM is relatively low as compared with the µXRF, the study identified less Zn signal for roots exposed to ZnS nanoparticles which were localised more at the cell wall (Chapter 6), these cell walls are considered an important sink for metal and a defence mechanism (Ringli, 2005). Storage mechanism in the cell wall involves precipitation in the cell wall or binding to cell wall components in the form of polygalacturonate, cellulose etc (Carrasco-Gil et al., 2013). Zn was present inside of the cells of roots exposed to ZnS and ZnO nanoparticles treatment,

suggesting that *Brassica juncea* absorbed these Zn nanoparticles. Bacteria were observed in the roots inoculated with $ZnSO_4$ than in Zn nanoparticles treatments (Chapter 6) suggesting that *P. brassicacearum* formed a close association with plant roots (Sessitsch et al., 2013; Prapagdee et al., 2013). XANES analysis on the Zn hotspot as identified by μXRF indicates Zn was strongly complexed by ligands indicating that changes in speciation were paramount in abating toxic Zn and *P. brassicacearum* induced speciation changes in the roots of *Brassica juncea* (Chapter 6). An important aspect of this study was to identify whether the reduction in Zn toxicity achieved through bacterial inoculation is associated with changes in Zn speciation in the plant root or whether speciation changes occured in the rhizosphere before plant uptake, explaining in part their toxicity ameliorating properties (Chapter 7). Results confirmed not much changes in speciation between bulk soil and rhizosphere but there was a significant change in the proportions of the different species between the rhizosphere and roots. Thus, these differences in Zn speciation in roots and rhizosphere soil are dependent on the form of Zn contamination. Moreover, *R. leguminosarum* modified Zn speciation across the rhizosphere and plant root depending on the form in which Zn was spiked in soil. In most cases, this study showed that in all Zn treatments, inoculation with *P. brassicacearum and R. leguminosarum* led to a significant improvement in plant growth due to the presence of cysteine, which ameliorated Zn toxicity.

8.2 Conceptual model for the combined role of Zn speciation and PGPB

A conceptual model of the role of Zn speciation in *Brassica juncea* exposed to different Zn species and in combination with plant growth promoting bacteria (PGPB) is presented in Figure 8.1, focusing on root and rhizospheric processes.

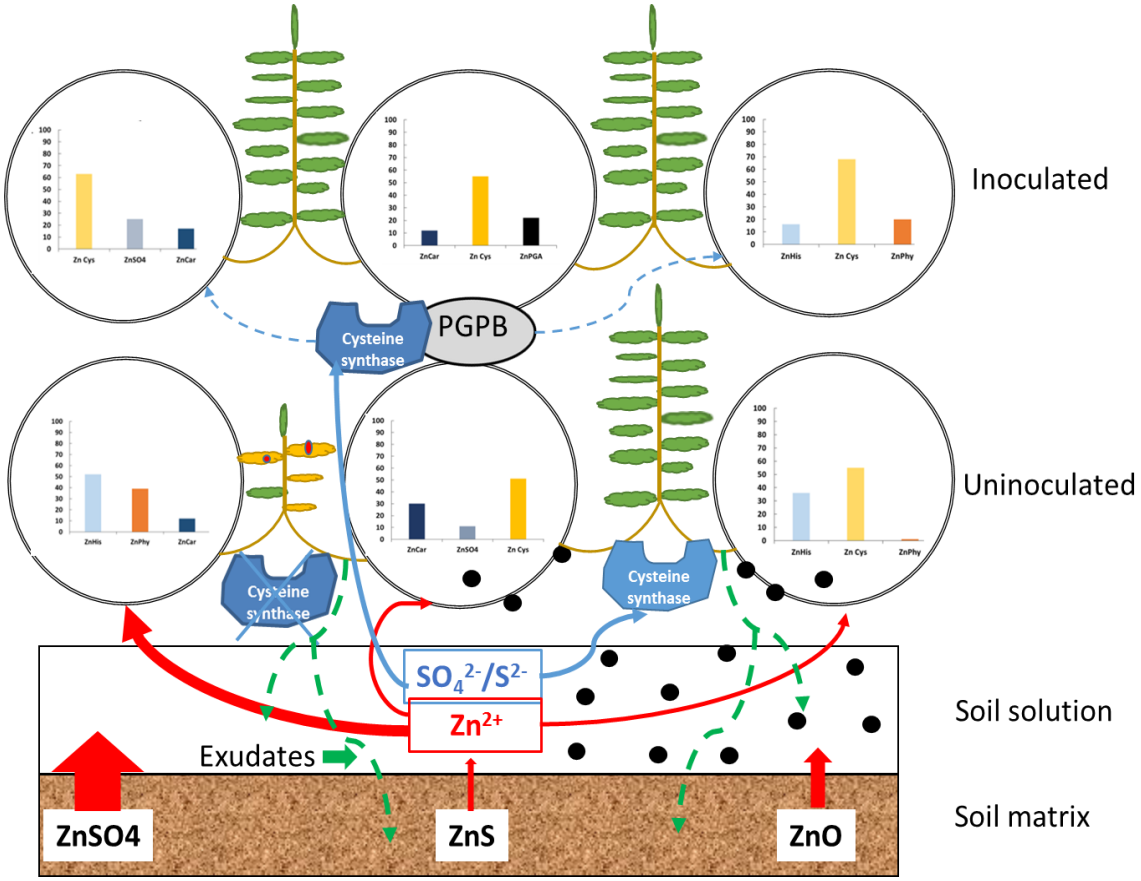


Figure 8.1: Conceptual model for zinc dynamics as revealed from pot experiments in which the form of Zn applied to soil where *Brassica juncea* was grown for 6 weeks was varied.

Seeds were planted either uninoculated or inoculated with plant growth promoting bacteria (PGPB) and speciation of Zn in the plant roots was carried out by XAS analysis. The model shows that Zn is mostly taken up as Zn^{2+}, in part facilitated by production of plant root exudates (large green dashed arrows), with cysteine synthesis (yellow bars) as the main mechanism of Zn detoxification but this is disabled (shown by blue cross under the enzyme "Cysteine synthase") at high Zn^{2+} concentrations (i.e. under soluble $ZnSO_4$). Inoculation with PGPB appears to restore or supplement cysteine production under all Zn treatments, possibly explaining the mechanism of Zn toxicity reduction by PGPB. Large circles represent roots within which are bar charts showing percent Zn speciation, black dots are nanoparticles. The model shares some attributes with that published by Adediran et al. (2016), which was based purely on $ZnSO_4$ contamination, but there are important differences that arise from varying the speciation of Zn supplied to soil. An

important aspect of the new model is the importance of solubility in controlling Zn bioavailability to plant roots, with higher dissolved Zn^{2+} from $ZnSO_4$, denoted by larger arrows, being the main determinant of toxicity, particularly when plants were not inoculated with bacteria. This is entirely consistent with existing models of metal bioavailability and phytotoxicity (Marschner, 1995; Alloway, 2008; Zhao et al., 2012; Mitra, 2015). Significantly, XANES analysis revealed major differences in the speciation of root Zn, with plants exposed to $ZnSO_4$ containing Zn in the form of Zn histidine and Zn pytate, whereas as those exposed to nanoparticlulate forms of Zn were dominated by Zn cysteine. Histidine has been shown to be produced as a defensive exudate upon exposure of plants to metals (e.g. Salt et al. 1999). Notably, it is also present in roots exposed to ZnO nanoparticles, where dissolved Zn concentrations are likely higher than in ZnS treatments (based on solubility data), further suggesting that its production is a defensive response to Zn^{2+} challenge.

Meanwhile, cysteine and its polymers (e.g glutathione) have been shown to be produced as a defence mechanism in response to heavy metal exposure (Zeng et al., 2011; Ma et al., 2015). Plants synthesise cysteine as part of their sulfur metabolism through a multiple enzymatic process that converts sulfate to sulfide which is then combined with O-acetylserine to form cysteine (Saito et al., 2000, Tavares et al., 2015). While Zn cysteine was detected in roots exposed to nanoparticulate forms of Zn when plants were not inoculated with bacteria, there was none in the $ZnSO_4$ treatments despite this treatment supplying the most sulfate. It is possible that the lack of cysteine in treatments is a direct result of the higher Zn^{2+} concentrations impairing the cysteine synthetic pathway but this hypothesis remains to be tested by detailed molecular level studies of the biochemistry of the reponse of *Brassica juncea* upon exposure to varying Zn^{2+} concentrations. Nevertheless, circumstantial evidence for this inference is that when inoculated with bacteria, roots exposed to $ZnSO_4$ appear to recover cysteine synthesis, and grow as well as those exposed to nanoparticulate Zn and/or controls without Zn addition, although the recovery may be due to additional cysteine synthesis by the plant growth promoting bacteria (Adediran et al., 2016).

Paradoxically, Zn cysteine was detected in roots exposed to ZnO where no sulfur is supplied to the soil. However, analysis of the soil showed that it contained a significant amount of sulfur (248.7 mg/kg), so this result is entirely consistent with the model of cysteine synthesis through sulfur metabolism. Lastly, the model captures the observation that in addition to soluble $Zn^{2+,}$ TEM revealed that Zn was also taken up in nanoparticulate form, albeit at much lower quantities. In addition to the model, the inoculation with PGPB potentially influenced Zn speciation through complexation which facilitated zinc tolerance and detoxification in *Brassica juncea* exposed to zinc contaminated soil.

8.3 Limitations and Recommendation

The limitations and recommendation for future works are summarised below.

In this study, the added Zn was homogenously mixed in soil after artificially spiking with different Zn species, which was used to influence plant available Zn in the pot experiment (Chapter 5-7). Several limitations may arise from the expected length of time at which different Zn species were added and equilibrated before planting. The time period (1 week) used for the incubation was 1 week, although similar time period used in this study was used in studying Zn responses of *Brassica juncea* grown in $ZnSO_4$ soils (Adediran et al., 2015). However, the equilibration time of applied ZnS and ZnO nanoparticles in soil is unknown. Therefore, a proposed study on how much time is needed for soil to equilibrate upon exposure to metal nanoparticles is required.

This study examined Zn bioavailability in soil pore water in all Zn treatments. 0.45 µm membrane filter was used as the suitable method for determining dissolved metal fraction (Harmsen, 2007) in pore water and was also used for Zn nanoparticles treated soil. Additionally, centrifugal ultrafiltration was also separately used in order to separate nanoparticulate Zn from the dissolved fraction. Dissolved Zn concentration in the pore water samples were measured using ICP-OES. However, there were no differences in Zn concentrations results measured with both filters. Owing to their unique properties, future studies should focus on characterisation of ZnS and ZnO nanoparticles, relevant exposure concentrations,

which will be vital in interpreting phytotoxicity results, understanding any comparisons made between studies as well as provide more environmentally relevant risk information. Development of new test guidelines and tools to differentiate between nano - specific toxicity and metal ion (Zn^{2+}) should also be a major focus in research as production of metal nanoparticles is expected to grow rapidly.

This research work was limited to a single hyperaccumulator species (*Brassica juncea*) carried out in the glasshouse under controlled conditions (light, temperature amongst others). A further extension of this research should be studying the effect of metal speciation in comparison with metal nanoparticles in a range of hyperaccumulating plant species under various soil conditions. These results should not be validated in greenhouse studies but rather in field site because results of controlled experimental studies may differ from those in the natural environment. For instance, root proliferation may be confined in pots under glasshouse conditions. However, roots may extend to deeper depth and to potentially less contaminated soil profile area in the field (Delorme et al., 2001).

This study showed that plant growth promoting bacteria were found effective in promoting plant growth and mitigating toxicity in the glasshouse soil based pot experiment. Inoculation of seeds with selected PGPB has the potential of improving phytoremediation by increasing metal uptake in plant and improving plant health in metal contaminated soil. Further research questions the successful use of such inoculum in the field with natural environmental conditions, bearing in mind the competitions of other present indigenous microorganisms.

The region around root and soil is a dynamic interface of several biogeochemical processes. An increased understanding is needed on the biological (microbial activity) and chemical (exudates) changes in the rhizosphere to identify key processes in mobilization of metal accumulation in plants. Also further study should focus on the role of organic ligands on metal toxicity and detoxification mechanisms in plant metabolisms.

8.3 Conclusion

This study provides new insights into the opportunities afforded by the interaction between hyperaccumulating plants and plant growth promoting bacteria when grown on Zn contaminated soil in gaining understanding on the role of Zn speciation. Zn behaviour in the soil- plant system and its phytoxicity is greatly influenced by Zn speciation. This study confirmed the main hypothesis that plant growth, toxicity, uptake by plants depended critically on form of Zn that *Brassica juncea* was exposed to, with soluble Zn salt ($ZnSO_4$) being more toxic than ZnS, ZnO nanoparticles. Addition of plant growth promoting bacteria alone and in combination with their plant host, can influence Zn solubility, availability to plants and ameliorate toxicity through complexation of Zn.

Phytoextraction - an environmentally sustainable technology that offers a less expensive method is promising because hyperaccumulator species have adaptive mechanisms for accumulating and tolerating high contaminant concentrations in their rhizosphere (Raskin et al., 1997). An understanding of the complex interactions including contaminated soil variables, plant –microbe interaction, and rhizosphere activities would allow the use of phytoextraction as friendly technology. However, phytoextraction must be approached as a multi-disciplinary research effort incorporating expertise from environmental engineers, soil microbiologist, plant biologist, agronomist, and biogeochemist to identify and solve metal contamination issues (Prassad et al., 2012).

Appendix

Appendix A- XANES spectra for plant roots, rhizosphere and bulk soil

The following graphs are XANES spectra of inoculated and uninoculated plant roots, rhizosphere and bulk soil treated with 600 mg Zn kg^{-1} of $ZnSO_4$ and ZnS nanoparticles (Chapter 7).

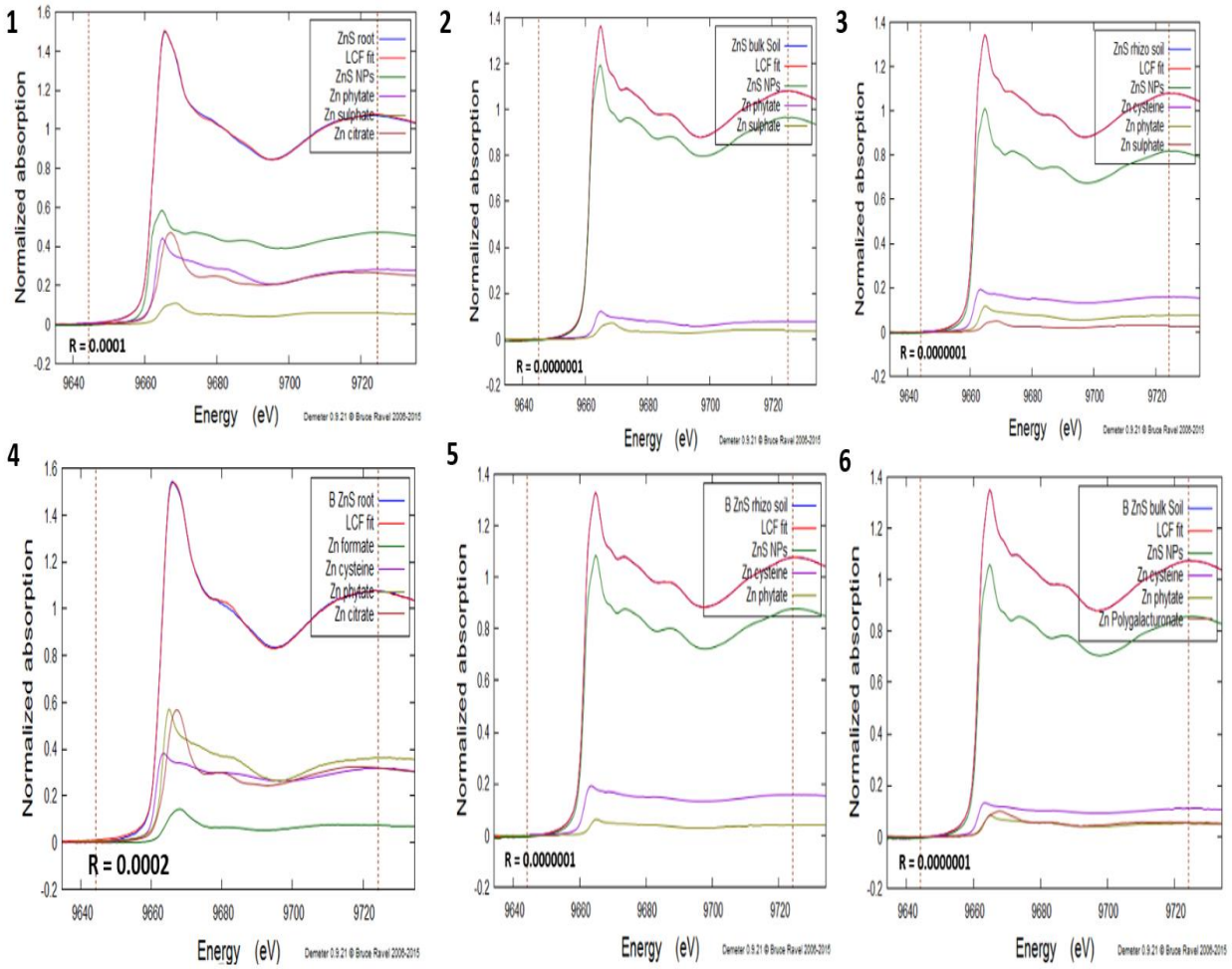


Figure A1.1: Result of Zinc K-edge XANES fitting of uninoculated *Brassica juncea* exposed to 600 mg/kg ZnS nanoparticles (1) roots, (2) bulk, (3) rhizosphere soil, *Brassica juncea* inoculated with *Rhizobium leguminosarum* (BZnS) in (4) roots (6) bulk soil (7) and rhizospheric soil. XANES spectra for each sample are the blue lines and LCF with zinc reference compounds spectra are donoted in red (lines). The goodness of fit is indicated by the R factor, with better fits indicated by lower R values.

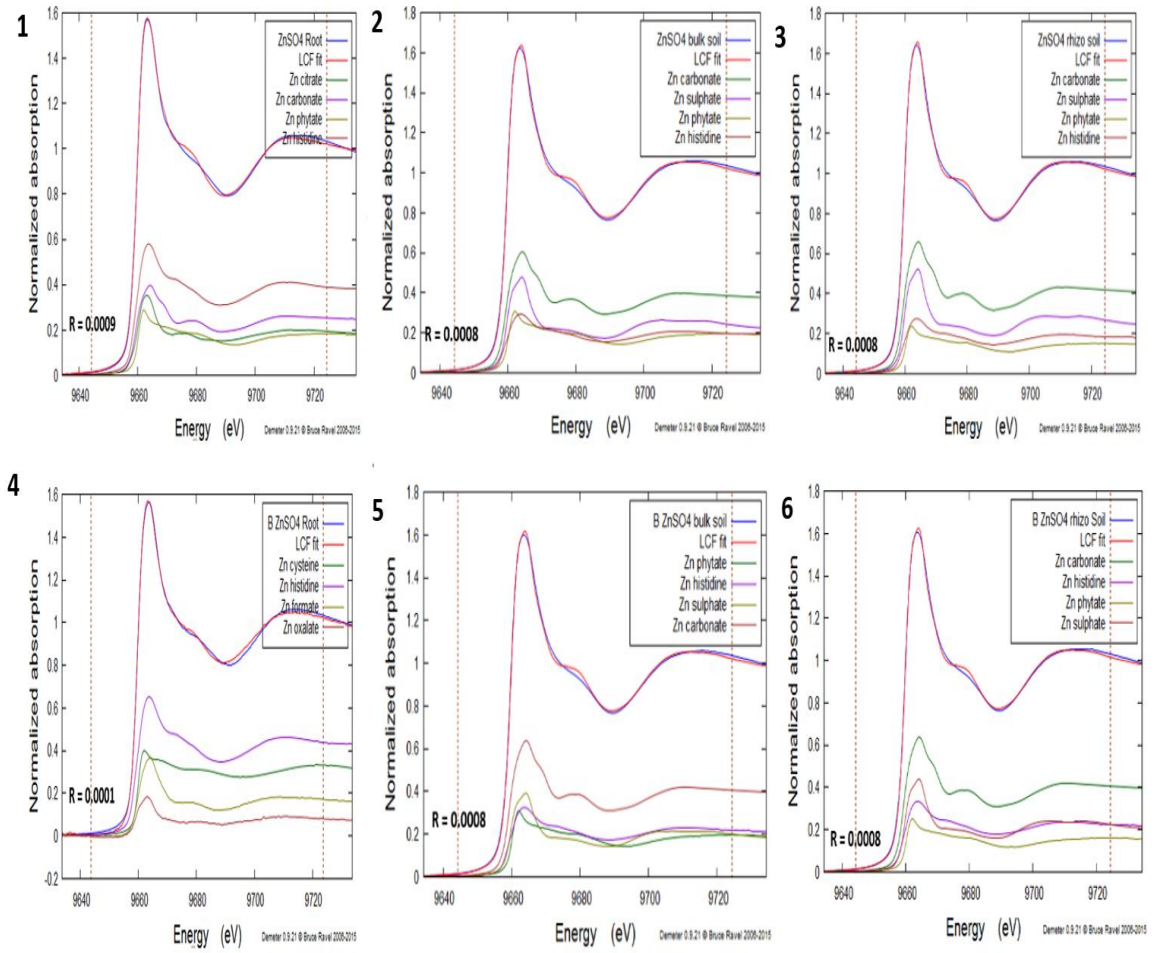


Figure A1.2: Result of Zinc K-edge XANES fitting of uninoculated *Brassica juncea* exposed to 600 mg / kg $ZnSO_4$ (1) roots, (2) bulk, (3) rhizosphere soil, *Brassica juncea* inoculated with *Rhizobium leguminosarum* (B $ZnSO_4$) in (4) roots (6) bulk soil (7) and rhizospheric soil. XANES spectra for each sample are the blue lines and LCF with zinc reference compounds spectra are donoted in red (lines). The goodness of fit is indicated by the R factor, with better fits indicated by lower R values.

Appendix B

B1.1: Anderson Darling's (AD) normality test for plant growth parameter Chapter 5. All treatment means are normally distributed (P > 0.05)

Parameters	Treatment	AD value	P value
Plant height			
	Control	0.135	0.948
	$ZnSO_4$	0.558	0.084
	ZnO	0.263	0.55
	ZnS	0.156	0.907
Number of leaves			
	Control	0.29	0.486
	$ZnSO_4$	0.243	0.619
	ZnO	0.381	0.272
	ZnS	0.341	0.354
Root biomass			
	Control	0.267	0.361
	$ZnSO_4$	0.189	0.631
	ZnO	0.19	0.629
	ZnS	0.398	0.119
Shoot biomass			
	Control	0.235	0.466
	$ZnSO_4$	0.39	0.128
	ZnO	0.228	0.493
	ZnS	0.19	0.629
Total chlorophyll content			
	Control	0.299	0.277
	$ZnSO_4$	0.488	0.061
	ZnO	0.487	0.062
	ZnS	0.485	0.06

B1.2: Anderson Darling's (AD) normality test for plant growth parameter in Chapter 6. All treatment means are normally distributed (P > 0.05)

Parameters	Treatments	AD value	P value
Plant height			
	Control	0.215	0.726
	$ZnSO_4$	0.352	0.328
	ZnO	0.14	0.94
	ZnS	0.264	0.548
	B1 control	0.193	0.809
	B1 $ZnSO_4$	0.182	0.846
	B1 ZnO	0.136	0.946
	B1 ZnS	0.182	0.845
	B2 control	0.182	0.845
	B2 $ZnSO_4$	0.208	0.754
	B2 ZnO	0.201	0.781
	B2 ZnS	0.169	0.883
Shoot biomass			
	Control	0.369	0.153
	$ZnSO_4$	0.334	0.208
	ZnO	0.19	0.628
	ZnS	0.449	0.079
	B1 Control	0.227	0.496
	B1 $ZnSO_4$	0.484	0.559
	B1 ZnO	0.293	0.294
	B1 ZnS	0.485	0.158
	B2 control	0.46	0.071
	B2 $ZnSO_4$	0.38	0.14
	B2 ZnO	0.293	0.294
	B2 ZnS	0.275	0.338
Root biomass			
	Control	0.266	0.364
	$ZnSO_4$	0.195	0.606
	ZnO	0.371	0.151
	ZnS	0.258	0.389
	B1 Control	0.249	0.418
	B1 $ZnSO_4$	0.271	0.35

	B1 ZnO	0.406	0.114
	B1 ZnS	0.199	0.588
	B2 Control	0.189	0.631
	B2 $ZnSO_4$	0.194	0.607
	B2 ZnO	0.233	0.475
	B2 ZnS	0.296	0.285

B1 represents *Rhizobium leguminosarum (bv) trifolii, and B2 represents Pseudomonas brassicacearum.*

B1.3: Anderson Darling's (AD) normality test for plant growth parameter in Chapter 7. All treatment means are normally distributed (P > 0.05)

Parameters	Treatment	AD value	P value
Plant height			
	Control	0.122	0.97
	$ZnSO_4$	0.588	0.068
	ZnS	0.141	0.937
	B1 Control	0.176	0.864
	B1 $ZnSO_4$	0.151	0.917
	B1 ZnS	0.134	0.95
Root length			
	Control	0.32	0.233
	$ZnSO_4$	0.259	0.384
	ZNS	0.259	0.384
	B1 Control	0.189	0.631
	B1 $ZnSO_4$	0.395	0.122
	B1 ZnS	0.27	0.352
Root biomass			
	Control	0.191	0.621
	$ZnSO_4$	0.23	0.487
	ZnS	0.212	0.536
	B1 Control	0.288	0.304
	B1 $ZnSO_4$	0.354	0.175
	B1 ZnS	0.23	0.487
Shoot biomass			
	Control	0.339	0.198
	$ZnSO_4$	0.446	0.081
	ZnS	0.47	0.066
	B1 Control	0.203	0.569
	B1 $ZnSO_4$	0.452	0.076
	B1 ZnS	0.278	0.329

B1 represents *Rhizobium leguminosarum (bv) trifolii*

References

Abbas-Zadeh, P., Saleh-Rastin, N., Asadi-Rahmani, H., Khavazi, K., Soltani, A., Shoary-Nejati, A.R., Miransari, M., 2010. Plant growth-promoting activities of fluorescent pseudomonads, isolated from the Iranian soils. Acta Physiologiae Plantarum 32, 281-288.

Abd-Elfattah, A.L.Y., Wada, K., 1981. Adsorption of lead, copper, zinc, cobalt, and cadmium by soils that differ in cation-exchange materials. Journal of Soil Science 32, 271-283.

Abou-Shanab, R.A., Ghanem, K., Ghanem, N., Al-Kolaibe, A., 2008. The role of bacteria on heavy-metal extraction and uptake by plants growing on multi-metal-contaminated soils. World Journal of Microbiology and Biotechnology 24, 253-262.

Achouak, W., Sutra, L., Heulin, T., Meyer, J.M., Fromin, N., Degraeve, S., Christen R., Gardan, L., 2000. Description of *Pseudomonas brassicacearum* sp. *nov*. and *Pseudomonas thivervalensis* sp. *nov*., two root-associated bacteria isolated from *Arabidopsis thaliana* and *Brassica napus*. International Journal of Systematic and Evolutionary Microbiology 50, 9-18.

Achouak, W., Conrod, S., Cohen, V., Heulin, T.T., 2004. Phenotypic variation of *Pseudomonas brassicacearum* as a plant root colonization strategy. Molecular Plant Microbe Interactions 17, 872-879.

Adamu, H., Luter, L., Lawan, M.M., Umar, B.A., 2013. Chemical speciation: a strategic pathway for insightful risk assessment and decision making for remediation of toxic metal contamination. Environment and Pollution 2, 92.

Adediran, G. A, Ngwenya, B.T., Mosselmans, J.F.W., Heal, K.V., Harvie, B.A., 2015. Mechanisms behind bacteria induced plant growth promotion and Zn accumulation in *Brassica juncea*. Journal of Hazardous Material 283, 490–499.

Adediran, G. A., Ngwenya, B. T., Mosselmans, J. F. W., Heal, K.V., 2016. Bacteria–zinc co-localization implicates enhanced synthesis of cysteine-rich peptides in zinc detoxification when *Brassica juncea* is inoculated with *Rhizobium leguminosarum*. New Phytologist 209, 280–293.

Adriano, D.C., 2001. Trace elements in terrestrial environments. Biogeochemistry, bioavailability and risks of metals. Springer-Verlag, New York. pp. 1-867.

Afzal, M., Yousaf, S., Reichenauer, T. G., Sessitsch, A., 2012. The inoculation method affects colonization and performance of bacterial inoculant strains in the phytoremediation of soil contaminated with diesel oil. International Journal of Phytoremediation 14, 35-47.

Ahemad, M., Khan, M.S., 2011. Response of greengram (Vigna radiata (L.) Wilczek) grown in herbicide-amended soil to inoculation with Bradyrhizobium sp. (vigna) MRM6. Journal of Agricultural Science and Technology 13, 1209-1222.

Ahemad, M., Kibret, M., 2014. Mechanisms and applications of plant growth promoting rhizobacteria: Current perspective. Journal of King Saud University Science 26, 1–20.

Ahmad, A., Ghufran, R., Zularisam, A.W., 2011. Phytosequestration of metals in selected plants growing on a contaminated Okhla industrial areas, Okhla, New Delhi, India. Water Air Soil Pollution 217, 255-266.

Al Chami, Z., Cavoski, I., Mondelli, D., Miano, T., 2013. Effect of compost and manure amendments on zinc soil speciation, plant content, and translocation in an artificially contaminated soil. Environmental Science and Pollution Research 20, 4766-4776.

Alhadrami, H.A., Mbadugha, L., Paton, G.I., 2016. Hazard and risk assessment of human exposure to toxic metals using in vitro digestion assay. Chemical Speciation and Bioavailability 28, 78-87.

Alamgir, M., 2016. The effects of soil properties to the extent of soil contamination with metals. In environmental remediation technologies for metal-contaminated soils. Springer Japan. pp. 1-19.

Alexander, M., 2000. Ageing, bioavailability and overestimation of risk from environmental pollutants. Environmental Science and Technology 34, 4259-4265.

Alford, É.R., Pilon-Smits, E.A., Paschke, M.W., 2010. Metallophytes—a view from the rhizosphere. Plant and Soil 337, 33-50.

Ali, H., Khan, E., Sajad, M.A., 2013. Phytoremediation of heavy metals—concepts and applications. Chemosphere 91, 869-881.

Ali, R., Qamer, S., Mateen, A., 2012. Effect of different concentrations of metals ions on Bacillus and Pseudomonas spp. isolated from industrial effluents of Faisalabad. International Journal of Veterinary Science 1, 98-103.

Allen, S.E., Grimshaw, H.M., Parkinson, J.A., Quarmby, C.L., 1974. Chemical analysis of ecological materials. Blackwell Scientific Publications.

Alloway, B.J., 1990. Heavy metals in soils. Blackie Academic and Professional.

Alloway, B. J., 1995. Heavy metals in soils. Chapman and Hall, London.

Alloway, B.J., Ayres, D.C., 1997. Chemical principles of environmental pollution. Chapman and Hal.

Alloway, B.J., 2004. Zinc in soils and crop nutrition. Brussels, Belgium: International Zinc Association.

Alloway, B.J., 2008. Zinc in soils and crop nutrition. International Zinc Association. Brussel and Paris.

Al-Shehbaz, I., Beilstein, M., Kellogg, E., 2006. Systematics and phylogeny of the *Brassicaceae* (Cruciferae): an overview. Plant System Evolution 259, 89-120.

Al-Shehbaz, I.A., 1973. The biosystematics of the genus *Thelypodium* (Cruciferae). Contributions from the Gray Herbarium of Harvard University 204, 3-148.

American Society for Testing and Materials (ASTM), 2012. Standard terminology relating to nanotechnology. ASTM E2456-06. Available online at: http://www.astm.org/Standards/E2456.htm. [Accessed 12 January 2016].

An, J., Zhang, M., Wang, S., J., 2008. Physical, chemical and microbiological changes in stored green asparagus spears as affected by coating of silver nanoparticles-PVP. LWT-Food Science and Technology 41, 1100-1107.

Andreazza, R., Bortolon, L., Pieniz, S., Giacometti, M., Roehrs, D.D., Lambais, M.A., Camargo, F.A.O., 2011. Potential phytoextraction and phytostabilization of perennial peanut on copper contaminated vineyard soils and copper mining waste. Biological Trace Element Research 143, 1729-1739.

Anju, M., Banerjee, D.K., 2011. Associations of cadmium, zinc, and lead in soils from a lead and zinc mining area as studied by single and sequential extractions Environmental Monitoring Assessment 176, 67–85.

Appel, O., Al-Shehbaz, I., 2003. Cruciferae. In: the families and genera of vascular plants. Springer –Verlag, Berlin. pp 75-174.

Araceli, P.S., Millan, R., Sierra, M.J., Alarcon, R., Garcia, P., Gil-Diaz, M., Vazquez, S., Lobo, M.C., 2012. Mercury uptake by *Silene vulgaris* grown on contaminated spiked soils. Journal of Environmental Management 95, 233–237.

Arduini, I., Godbold, D. I., Onnis, A., 1994. Cadmium and copper change root growth and morphology of *Pinus pinea* and *Pinus pineaster* seedlings. Physiologia Plantarum 92, 675-680.

Arnold, R.E., Hodson, M.E., Comber, S., 2007. Does speciation impact on Cu uptake by, and toxicity to the earthworm Eisenia fetida? European Journal of Soil Biology 43, S230-S232.

Arnon, D.I., 1949. Copper enzyme in isolated chloroplast: Polyphenol oxidase in *Beta vulgaris*. Plant Phyisology 24, 1-15.

Arruda, S.C.C., Silva, A.L.D., Galazzi, R.M., Azevedo, R.A., Arruda, M.A.Z., 2015. Nanoparticles applied to plant science: A review. Talanta 131, 693-705.

Ashraaf, M.A., Maah, M.J., Yusoff, I., 2012. Chemical speciation and potential mobility of heavy metal of heavy metals in the soil of former tin mining catchment. The Scientific World Journal 11.

Baath, E., 1992. Measurement of heavy metal tolerance of soil bacteria using thymidine incorporation into bacteria extracted after homogenization-centrifugation. Soil Biology and Biochemistry 24, 1167-1172.

Badri, D.V., Weir, T.L., Van der Lelie, D., Vivanco, J.M., 2009. Rhizosphere chemical dialogues: Plant-microbe interactions. Current Opinion in Biotechnology 20, 642–650.

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S., Vivanco, J.M., 2006. The role of root exudates in rhizosphere interactions with plants and other organisms. Annual Review of Plant Biology 57, 233–266.

Baker, A.J.M., Walker, P.L., 1989. Ecophysiology of metal uptake by tolerant plants. Heavy metal tolerance in plants. CRC Publisher, Boca Raton, FL. pp. 155-177.

Baker, A.J.M., Reeves, R.D.A., Hajar, S.M., 1994. Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. & C. Presl (Brassicaceae). New Phytologist 127, 61–68.

Baker, A.J.M., McGrath, S.P., Reeves R.D., Smith J.A.C., 2000. Metal hyperaccumulator plants: A review of the ecology and physiology of a biochemical resource for phytoremediation of metal-polluted soils. Phytoremediation of contaminated soil and water. Lewis Publisher, Boca Raton, FL. pp. 85–107.

Bakshi, S., He, Z.L., Harris, W.G., 2015. Natural Nanoparticles: Implications for Environment and Human Health, Critical Reviews in Environmental Science and Technology 45, 861-904.

Banasova, V., Horak, O., 2008. Heavy metal content in *Thlapsi caerulescens* J. at C. fresh growing on metalliferous and non metalliferous soils in Central Slovakia. International Journal of Environment and Pollution 33, 133-145.

Bandyopadhyay, S., Plascencia-Villa, G., Mukherjee, A., Rico, C.M., José-Yacamán, M., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2015. Comparative phytotoxicity of ZnO NPs, bulk ZnO, and ionic zinc onto the alfalfa plants symbiotically associated with Sinorhizobium meliloti in soil. Science of the Total Environment 515, 60-69.

Barak, P., Helmke, P.A., 1993. The chemistry of zinc. In zinc in soils and plants. Kluwer Academic Publishers, Dordrecht. pp. 93-102.

Barnes, R.J., Riba, O., Gardner, M.N., Scott, T.B., Jackman, S.A., Thompson, I.P., 2010. Optimization of nano-scale nickel /iron particles for the reduction of high concentration chlorinated aliphatic hydrocarbon solutions. Chemosphere 79, 448-454.

Barney, D.L., 2003. Effects of light, surface sterilization, and fungicides on the germination of Black Huckleberry seeds. Small Fruits Review 2, 73-80.

Barrow, N.J., 1993. Mechanisms of reaction of zinc with soil and soil components. In Zinc in soils and plants. Springer Netherlands. pp. 15-31.

Barrow, N.J., Whelan, B.R., 1998. Comparing the effects of pH on the sorption of metals by soil and by goethite, and on uptake by plants. European Journal of Soil Science 49, 683-692.

Bashan, Y., Holguin, G., 1997. *Azosprillum*-plant relationships: environmental and physiological advances (1990-1996). Canandian Journal of Microbiology 43, 103-121.

Batayneh, A.T., 2012. Toxic (aluminum, beryllium, boron, chromium and zinc) in groundwater: health risk assessment. International Journal of Environmental Science Technology 9, 153–162.

Batty, L.C., Anslow, M., 2008. Effect of a polycyclic aromatic hydrocarbon on the phytoremediation of zinc by two plant species (*Brassica juncea* and *Festuca arundinacea*). International Journal of Phytoremediation 10, 236-251.

Bauddh, K., Singh, R.P., 2012. Growth, tolerance efficiency and phytoremediation potential of *Ricinus communis (L.)* and *Brassica juncea* (L.) in salinity and drought affected cadmium contaminated soil. Ecotoxicology and Environmental Safety, 85, 13-22.

Beath, O.A., Eppsom, H.F., Gilbert, G.S., 1937. Selenium distribution in and seasonal variation of vegetation type occurring on seleniferious soils. Journal of the American Pharmaceutical Association 26, 394-405.

Belimov, A.A., Safronova, V.I., Sergeyeva, T.A., Egorova, T.N., Matveyeva, V.A., 2001. Characterisation of plant growth-promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1- carboxylate deaminase. Canadian Journal of Microbiology 47, 642–652.

Bell, B.C., 1993. Basic properties of soils. Australian Society of Soil Science Inc., Queensland, Brisbane, Australia. pp. 33-54.

Benadives, M.P., Gallego, S.M, Tomaro, M.L., 2005. Cadmium toxicity in plants. Brazilian Journal of Plant Physiology 17, 1- 18.

Bhargava, A., Carmona, F.F., Bhargava, M., Srivastava, S., 2012. Approaches for enhanced phytoextraction of heavy metals. Journal of Environmental Management 105, 103-120.

Bhattacharyyaa, K.G., Guptab, S.S., 2008. Adsorption of a few heavy metals on natural and modified kaolinite and montmorillonite: A review. Advances in Colloid and Interface Science 140, 114-131.

Bhuiyan, M.S., Raman, A., Hodgkins, D., Mitchell, D., Nicol, H., 2016. An ex situ salinity restoration assessment using legume, saltbush, and grass in Australian soil. Clean–Soil, Air, Water. DOI: 10.1002/clen.201500214.

Birbaum, K. Brogioli, R., Schellenberg, M., Martinoia, E. Stark, W. J., Gunther, D. Limbach, L. K., 2010. No evidence for cerium dioxide nanoparticle translocation in maize plants. Environmental Science and Technology 44, 8718−8723.

Bjerre, G.K., Schierup, H.H., 1985. Influence of waterlogging on availability and uptake of heavy metals by oat grown in different soils. Plant and Soil 88, 45–56.

Black, A., McLaren, R.G., Reichman, S.M., Speir, T.W., Condron, L.M., 2011. Evaluation of soil metal bioavailability estimates using two plant species (*L. perenne* and *T. aestivum*) grown in a range of agricultural soils treated with biosolids and metal salts. Environmental Pollution 159, 1523-1535.

Bluskov, S., Arocena, J. M., Omotoso, O. O., Young, J. P., 2005. Uptake, distribution, and speciation of chromium in *Brassica juncea*. International Journal of Phytoremediation 7, 153–165.

Bluskov, S., Arocena, J. M., Omotoso, O. O., Young, J. P., 2005. Uptake, distribution, and speciation of chromium in *Brassica juncea*. International Journal of Phytoremediation 7, 153–165.

Bokhari, S.Y., Ahmad, H.I, Mahmood-Ul-Hassan, M., Mohammad, A., 2016. Phytoremediation potential of Lemna minor L. for heavy metals. International Journal of Phytoremediation, 18, 25-32.

Bonner, E.R., Cahoon, R.E., Knapke, S.M., Jez, J.M., 2005. Structural and functional analysis of o-acetylserine sulfhydrylase from Arabidopsis thaliana The Journal of Biological Chemistry 280, 38803–38813.

Bonnet, M., Camares, O., Veisseire, P., 2000. Effects of zinc and influence of Acremonium lolii on growth parameters, chlorophyll a fluorescence and antioxidant enzyme activities of ryegrass (*Lolium perenne L. cv Apollo*). Journal of Experimental Botany 51, 945-953.

Bourg, A.C.M., 1988, Metal in aquatic and terrestrial systems: Sorption, speciation, and mobilization. In Chemistry and biology of solid waste: Berlin, Springer-Verlag. pp. 3-32.

Boussen, S., Soubrand, M., Bril, H., Ouerfelli, K., Abdeljaouad, S., 2013. Transfer of lead, zinc and cadmium from mine tailings to wheat (*Triticum aestivum*) in carbonated Mediterrenean (Northern Tunisia) soils. Geoderma 192, 227-236.

Boxall, A., Chaudhry, Q., Sinclair, C., Jones, A., Aitken, R., Jefferson, B., Watts, C., 2007. Current and future predicted environmental exposure to engineered nanoparticles. Central Science laboratory, Department of the Environment and Rural Affairs, London, UK. Available at: <http:// www.defra.gov.uk>. [Accessed 8 August 2012].

Bradl, H.B, Kim, C., Kramar, U., Stuben, D., 2005. Interactions of heavy metals. In heavy metals in the environment: origin, interaction and remediation. Elsevier Ltd., London. pp. 28-164.

Brady, J.E., Humiston, G.E., Heikkinen, H., 1983. General chemistry: principles and structure. Wiley. pp 671.

Brady, N.C., Weil, R.R., 2002. The nature and properties of soils. Prentice Hall, New Jersey. pp. 960.

Braud, A., Jezequel, K., Bazot, S., Lebeau, T., 2009. Enhanced phytoextraction of an agricultural Cr, Hg- and Pb-contaminated soil by bioaugmentation with siderophore producing bacteria. Chemosphere 74, 280-286.

Brayner, R., Ferrari-Iliou, R., Brivois, N., Djediat, S., Benedetti, M.F., Fievet, F., 2006. Toxicological impact studies based on *Escherichia coli* bacteria in ultrafine ZnO nanoparticles colloidal medium. Nanotechnology Letters 6, 866–870.

Brennan, R. F. Bolland, M. D. A., 2006. Zinc sulphate is more effective at producing wheat shoots than zinc oxide in an alkaline soil but both sources are equally effective in an acid soil. Australian Journal of Experimental Agriculture 46, 1615–1620.

Broadley, M.R., White, P.J., Hammond, J.P., Zelko, I., Lux, A., 2007. Zinc in plants. New Phytologist 173, 677–702.

Brooks, R.R., Chambers, M.F., Nicks, L.J., Robinson, B.H., 1998. Phytomining. Trends in Plant and Science 1, 359-362.

Brown, G.E., Calas, G., Waychunas, G.A., Petiau, J., 1988. X-ray absorption spectroscopy and its applications in mineralogy and geochemistry. Reviews in Mineralogy and Geochemistry 18, 431-512.

Brown, G.E., Sturchio, N.C., 2002. An overview of synchrotron radiation applications to lowtemperature geochemistry and environmental science. Reviews in Mineralogy and Geochemistry 49, 1-115.

Brown, S.L., 1994. Phytoremediation potential of *Thlaspi caerulescens* and *bladder campion* for zinc- and cadmium-contaminated soil. Journal of Environmental Quality 23, 1151–1157.

Brown, S.L., Henry, C.L., Chaney, R., Compton, H., De Volder, P.S., 2003. Using municipal biosolids in combination with other residuals to restore metal-contaminated mining areas. Plant Soil 249, 203-215.

Bruder-Hubscher, V., Lagarde, F., Leroy, M.J.F., Coughanowr, C., Enguehard, F., 2002. Application of a sequential extraction procedure to study the release of elements from municipal solid waste incineration bottom ash. Analytica Chimica Acta 451, 285-295.

Brummer, G., Tiller, K.G., Herms, U., Clayton, P.M., 1983. Adsorption-desorption and/or precipitation-dissolution processes of zinc in soils. Geoderma 31, 337–354.

Brunetti, G., Soler-Rovira, P., Farrag, K., Senesi, N., 2009. Tolerance and accumulation of heavy metals by wild plant species grown in contaminated soils in Apulia region, Southern Italy. Plant Soil 318, 285-298.

Burd, G.I., Dixon, D.G., Glick, B.R., 2000. Plant growth promoting bacteria that decreases heavy metal toxicity in plants. Canadian Journal of Microbiology 46, 237–245.

Cabiscol, E., Tamarit, J., Ros, J., 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. International Microbiology 3, 3-8.

Cakmak, I., Marschner, H., Bangerth, F., 1989. Effect of zinc nutritional status on growth, protein metabolism and levels of indole-3-acetic acid and other phytohormones in bean (*Phaseolus vulgaris L*.). Journal of Experimental Botany 40, 405-412.

Cakmak, I., 2000. Role of zinc in protecting plant cels from reactive oxygen species. New Phytologist 146, 185–205.

Calas, G., Brown, G.E., Waychunas, G.A., Petiau, J., 1987. X-ray absorption spectroscopic studies of silicate glasses and minerals. Physics and Chemistry of Minerals 15, 19-29.

Calheiros, C.S.C., Rangel, A.O.S.S., Castro, P.M.L., 2008. The effects of tannery wastewater on the development of different plant species and chromium accumulation in *phragmites australis*. Archives of Environmental Contamination and Toxicology 55, 404-414.

Cappuyns, V., Swennen, R., Niclaes, M., 2007. Application of the BCR sequential extraction scheme to dredged pond sediments contaminated by Pb–Zn mining: a combined geochemical and mineralogical approach. Journal of Geochemical Exploration 93, 78-90.

Carey, A., Lombi, E., Donner, E., De Jonge, M.D., Pushon, T., Jackson, B., Guerinot, M.L., Price, A.H., 2012. A review of recent developments in speciation and location of arsenic and selenium in rice grain. Analytical and Bioanalytical Chemistry 402, 3275-3286.

Carrasco-Gil, S., Siebner, H., LeDuc, D.L., Webb, S.M., Millan, R., Andrew, J.C., Hernandez, L.E., 2013. Mercury localization and speciation in plants grown hydroponically or in a natural environment. Environmental Science and Technology 47, 3082-3090.

Castillo-Michel, H., Hernandez-Viezcas, J.A., Servin, A., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2012. Arsenic localization and speciation in the root–soil interface of the desert plant *Prosopis juliflora-velutina*. Applied Spectroscopy 66, 719-727.

Cavallaro, N., McBride, M.B., 1984. Zinc and copper sorption and fixation by an acid soil clay: Effect of selective dissolutions. Soil Science Society of America Journal 48, 1050-1054.

Certini, G., Ugolini, F.C., 2013. An updated, expanded, universal definition of soil. Geoderma 192, 378-379.

Chakrabarti, P., 1990. Geometry of interaction of metal ions with histidine residues in protein structures. Protein Engineering 4, 57-63.

Champagne, E.T., Fisher, M.S., 1990. Binding differences of Zn (II) and Cu (II) ions with phytate. Journal of Inorganic Biochemistry 38, 217-223.

Chaney, R.L., 1983. Plant uptake of inorganic waste constituents. Noyes Data Corporation, Park Ridge, NJ, USA. pp. 50-76.

Chaney, R.L., Mielke, H.W., Sterrett, S.B., 1989. Speciation, mobility and bioavailability of soil lead. Environmental Geochemistry and Health 11, 105-129.

Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., Baker, A.J. 1997. Phytoremediation of soil metals. Current Opinion in Biotechnology 8, 279-284.

Chaney, R.L., Broadhurst, C.L., Centofanti, T., 2010. Phytoremediation of soil trace elements. Trace elements in soils. John Wiley and Sons, Chichester. pp. 311–352.

Chang, Y., Zhang, M., Xia, L., Zhang, J., Xing, G., 2012. The toxic effects and mechanisms of CuO and ZnO nanoparticles. Materials 5, 2850-2871.

Chaurasia, S., Karwariya, A., Gupta, A.D., .2013. Effect of cement industry pollution on chlorophyll content of some crops at Kodinar, Gujarat, India Sadhana. Proceedings of the International Academy of Ecology and Environmental Sciences 3, 288-295.

Chavan, A.S., Banerjee, N.K., 1980. Fe-Zn interaction in a black loamy soil as studied on rice crop. Indian Society of Soil Science 28, 203–205.

Chen, B.D., Li, X.L., Tao, H.Q., Christie, P., Wong, M.H., 2003. The role of arbuscular mycorrhizal in zinc uptake by red clover growing in a calcareous soil spiked with various quantities of zinc. Chemosphere 50, 839–846.

Chen, B.D., Zhu, Y.G., Duan, J., Xiao, X.Y., Smith, S.E., 2007. Effects of the arbuscular mycorrhizal fungus *Glomus mosseae* on growth and metal uptake by four plant species in copper mine tailings. Environmental Pollution 147, 374-80.

Chen, M., Ma, L.Q., 2001. Comparison of three aqua regia digestion. Methods for twenty Florida soils. Soil Science Society American Journal 65, 491–499.

Chen M., 2010. analytical integration procedures for the derivation of risk-based generic assessment criteria for soil, human and ecological risk assessment: An International Journal 16, 1295-131.

Chen, R., Ratnikova, T.A., Stone, M.B., Lin, S., Lard, M., Huang, G., Hudson, J.S., Ke, P.C., 2010. Differential uptake of carbon nanoparticles by plants and mammalian cells. Small 6, 612-617.

Chen, Y., Li, X., Shen, Z., 2004. Leaching and uptake of heavy metal by ten different species of plants during an EDTA-assisted phytoextraction process. Chemosphere 57, 187–196.

Chen, Z.J., Sheng, X.F., He, L.Y., Huang, Z., Zhang, W.H., 2013. Effects of root inoculation with bacteria on the growth, Cd uptake and bacterial communities associated with rape grown in Cd-contaminated soil. Journal of Hazardous Materials 244, 709–717.

Cheng, Z., Lee, L., Dayan, S., Grinshtein, M., Shaw, R., 2011. Speciation of heavy metals in garden soil: evidences from selective and sequential chemical leaching. Journal of Soils and Sediments 11, 628-638.

Chiang, K.Y., Wang, Y.N., Wang, M.K., Chiang, P.N., 2006. Low-molecular-weight organic acids and metal speciation in rhizosphere and bulk soils of a temperate rain forest in Chitou, Taiwan. Taiwan Journal for Science 21, 327-337.

Chopin, E.I.B., Alloway, B.J., 2007. Distribution and mobility of trace elements in soils and vegetation around the mining ane smelting areas of Tharsis, Riotinto and Huelva, Iberian pyrite Belt, SW Spain. Water, Air, and Soil Pollution 182, 245-261.

Chopin, E.I.B., Marin, B., Mkoungafoko, R., Rigaux, A., Hopgood, M.J., Delannoy, E., Cancès, B., Laurain, M., 2008. Factors affecting distribution and mobility of trace elements, Cu, Pb, Zn in a perennial grapevine, *Vitis vinifera* L. in the champagne region of France. Environmental Pollution 156, 1092-1098.

Chopra, I., 2007. The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern? Journal of Antimicrobial Chemotherapy 59, 587–590.

Chuan, M.C., Shu, G.Y., Liu, J.C., 1996. Solubility of heavy metals in a contaminated soil: Effects of redox potential and pH. Water, Air and Soil Pollution 90, 543–556.

Clark, R.B., Baligar, V.C., 2000. Acidic and alkaline soil constraints on plant mineral nutrition. Plant-Environment Interactions. Marcel Dekker, New York. pp. 133-177.

Clemens, S., 2010. Zn - a versatile player in plant cell biology. Cell biology of metals and nutrients. Springer, Berlin Heidelberg. pp. 281–298.

Clever, H.L., Derrick, M.E., Johnson, S.A., 1992. The solubility of some sparingly soluble salts of zinc and cadmium in water and in aqueous-electrolyte solutions. Journal of Physics and Chemical Reference Data 21, 941–1004.

Clough, R., Lindsay, R., Drennar, H., Harrington, C.F., Hill, S.J., Tyson, J.F., 2012. Atomic spectrometry Update: Elemental speciation. Journal of Analytical Atomic Spectrometry 27, 1185-1224.

Cobbett, C.S., 2000. Phytochelatins and their roles in heavy metal detoxification. Plant Physiology 123, 825-832.

Compant, S., Duffy, B., Nowak, J., Clement, C., Barka, E.A., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology 71, 4951–4959.

Cornelis, G., Hund-Rinke, K., Kuhlbusch, T., Van den Brink, N., Nickel, C., 2014. Fate and bioavailability of engineered nanoparticles in soils: a review. Critical Reviews in Environmental Science and Technology 44, 2720-2764.

Corredor, E., Testillano, P.S., Coronado, M.J., González-Melendi, P., Fernández-Pacheco, R., Marquina, C., Ibarra, M.R., De la Fuente, J.M., Rubiales, D., Pérez-de-Luque, A., Risueño, M.C., 2009. Nanoparticle penetration and transport in living pumpkin plants: in situ subcellular identification. BMC Plant Biology 9, 1-45.

Cortazar, B., Koydemir, H.C., Tseng, D., Feng, S., Ozcan, A., 2015. Quantification of plant chlorophyll content using google glass. Lab on a Chip 15, 1708-1716.

Covelo, E.F., Vega, F.A., Andrade, M.L., 2008. Sorption and desorption of Cd, Cr, Cu, Ni, Pb and Zn by a fibric histosol and its organo-mineral fraction. Journal of Hazardous Materials 159, 342-347.

Croes, S., Weyens, N., Janssen, J., Vercampt, H., Colpaert, J.V., Carleer, R., Vangronsveld, J., 2013. Bacterial communities associated with *Brassica napus* L. grown on trace element-contaminated and non-contaminated fields: a genotypic and phenotypic comparison. Microbial Biotechnology 6, 371–384.

Cuong, D., Obbard, J.P., 2006. Metal speciation in coastal marine sediments from Singapore using a modified BCR-sequential extraction procedure. Applied Geochemistry 21, 1335-1346.

Curl, E.A., Truelove, B., 1986. The Rhizosphere. SpringerVerlag, Berlin.

Dalvi, A.A., Bhalerao, S.A., 2013. Response of plants towards heavy metal toxicity: an overview of avoidance, tolerance and uptake mechanism. Annals of Plant Sciences 2, 362–368.

Das, S., Wolfson, B.P., Tetard, L., Tharkur, J., Bazata, J., Santra, S., 2015. Effect of N-acetyl cysteine coated CdS: Mn/ZnS quantum dots on seed germination and seedling growth of snow pea (Pisum sativum L.): imaging and spectroscopic studies. Environmental Science: Nano 2, 203-212.

Da Silva, J.J.F., Williams, R.J.P., 1991. Zinc: Lewis acid catalysis and regulation. Clarendon Press, Oxford. pp. 299-318.

Davidson, C.M., Urquhart, G.J., Ajmone-Marsan, F., Biasioli, M., da Costa Duarte, A., Dıaz-Barrientos, E., Grcman, H., Hossack, I., Hursthouse, A.S., Madrid, L., Rodrigues, S., Zupan, M., 2006. Fractionation of potentially toxic elements in urban soils from five European cities by means of a harmonised sequential extraction procedure. Analytical Chimca Acta 565, 63–72.

Davies, B.E., Jones, L.H.P., 1988. Micronutrients and toxic elements. Russell's soil conditions and plant growth. John Wiley & Sons; Interscience, New York, NY, USA, eleventh edition. pp. 780–814.

Davis, J.G., Parker, M.B., 1993. Zinc toxicity symptom development and partioning of biomass and zinc in peanut plants. Journal of Plant Nutrition 16, 2353–2369.

De-Bashan, L.E., Hernandez, J.P, Bashan, Y., 2011. The potential contribution of plant growth- promoting bacteria to reduce environmental degradation – A comprehensive evaluation. Applied Soil Ecology 61, 171-189.

Dede, G., Ozdemir, S., Dede, O.H., 2012. Effect of soil amendments on phytoextraction potential of *Brassica juncea* growing on sewage sludge. International Journal of Environmental Science and Technology 9, 559–564.

Deletic, A., Orr, D.W., 2005. Pollution build up on road surfaces. Journal of Environment Engineering 131, 49-59.

Delorme, T.A., Gagliardi, J.V., Angle, J.S., Chaney, R.L., 2001. Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. and the non-metal accumulator *Trifolium pratense* L. on soil microbial populations. Canadian Journal of Microbiology 47, 773-776.

Dessaux, Y., Grandclément, C., Faure, D., 2016. Engineering the Rhizosphere. Trends in Plant Science 21, 266-278.

Dessureault-Rompré, J., Nowack, B., Schulin, R., Tercier-Waeber, M.L., Luster, J., 2008. Metal solubility and speciation in the rhizosphere of Lupinus albus cluster roots. Environmental Science and Technology 42, 7146-7151.

Dhas, S.P., Shiny, P.J., Khan, S., Mukherjee, A., Chandrasekaran, N., 2013. Toxic behaviour of silver and zinc oxide nanoparticles on environmental microorganisms. Journal of Basic Microbiology 53, 1–12.

Dhir, B., Sharmila, P., Pargha- Sarad, P., 2008. Photosynthetic performance of *Salvinia natans* exposed to chromium and zinc rich wastewater. Brazilian Journal of Plant Physiology 20, 61-70.

Di Bonito, M., 2005. Trace elements in soil pore water: A comparison of sampling methods. Ph.D. Thesis, University of Nottingham. pp 299.

Di Bonito, M., Breward, N., Crout, N., Smith, B., Young, S., 2008. Overview of selected soil pore water extraction methods for the determination of potentially toxic elements in contaminated soils: operational and technical aspects. Environmental Geochemistry: Site Characterization, Data Analysis and Case Histories. Amsterdam. pp. 213–249.

Dietz, K.J., Herth, S., 2011. Plant nanotoxicology. Trends in Plant Science 16, 582–589.

Dimkpa, C., Svatos, A., Dabrowska, P., Schmidt, A., Boland, W., Kothe, E., 2008a. Involvement of siderophores in the reduction of metal-induced inhibition of auxin synthesis in Streptomyces spp. Chemosphere 74, 19–25.

Dimkpa, C., Svatos, A., Merten, D., Büchel, G., Kothe, E., 2008b. Hydroxamate siderophores produced by *Streptomyces acidiscabies* E13 bind nickel and promote

growth in cowpea (*Vigna unguiculata L*.) under nickel stress. Canadian Journal of Microbiology 54, 163–172.

Dimkpa, C., Merten, D., Svatos, A., Buchel, G., Kothe, E., 2009a. Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. Soil Biology and Biochemistry 41, 154–162.

Dimkpa, C., Merten, D., Svatos, A., Büchel, G., Kothe, E., 2009b. Siderophores mediate reduced and increased uptake of cadmium by Streptomyces tendae F4 and sunflower (*Helianthus annuus*) respectively. Journal of Applied Microbiology 107, 1687–1696.

Dimkpa, C.O., McLean, J.E., Latta, D.E., Manangón, E., Britt, D.W., Johnson, W.P., Boyanov, M.I., Anderson, A.J., 2012. CuO and ZnO nanoparticles: phytotoxicity, metal speciation, and induction of oxidative stress in sand-grown wheat. Journal of Nanoparticle Research 14, 1-15.

Dimkpa, C.O., Latta, D.E., McLean, J.E., Britt, D.W., Boyanov, M.I., Anderson, A.J., 2013. Fate of CuO and ZnO nano-and microparticles in the plant environment. Environmental Science and Technology 47, 4734-4742.

Dimkpa, O.C., Hansen, T., Stewart, J., McLean, J.E., Britt, W.D., Anderson, A.J., 2014. ZnO nanoparticles and root colonization by a beneficial pseudomonad influence essential metal responses in bean (*Phaseolus vulgaris*). Nanotoxicology 9, 271-278.

Ding, S., Hu, X., Wu, X., Yin, D., 2010. Metal contents and fractionation in contaminated soil after column leaching using [S, S]-EDDS. Chemical Speciation and Bioavailability 22, 247-255.

Disante, K.B., Fuentes, D., Cortina, J., 2011. Response to drought of Zn-stressed *Quercus suber L.* seedlings. Environmental and Experimental Botany 70, 96-103.

DoE., 1996. Department of the Environment. Code of Practice for Agriculture Use of Sewage Sludge. DoE Publication.

Doelman, P., Haanstra, L., 1984. Short-term and long-term effects of cadmium, chromium, copper, nickel, lead and zinc on soil microbial respiration in relation to abiotic soil factors. Plant and soil 79, 317-327.

Donner, E., Broos, K., Heemsbergen, D., Warne, M.S.J., McLaughlin, M.J., Hodson, M.E., Nortcliff, S., 2010. Biological and chemical assessments of zinc ageing in field soils. Environmental Pollution 158, 339-345.

Donner, E., De Jong, M.D., Kopittke, P.M., Lombi, E., 2013. Mapping elemental distributions in plant tissue using synchrotron x-ray fluorescence techniques. Methods in Molecular Biology 953, 143-159.

Doty, S.L., Shang, T.Q., Wilson, A.M., Moore, A.M., Strand, S.E., Oda, C., Gordon, M.P., 2003. Metabolism of the halogenated hydrocarbons, TCE and EDB, by the tropical leguminous tree, *Leucocaena leucocephala*. Water Research 37, 441-449.

Doty, S.L., 2008. Tansley Review: Enhancing phytoremediation through the use of transgenics and endophytes. New Phytologist 179, 318–333.

Du, W., Sun, Y., Ji, R., Zhu, J., Wu, J., Guo, H., 2011. TiO_2 and ZnO nanoparticles negatively affect wheat growth and soil enzyme activities in agricultural soil. Journal of Environmental Monitoring 13, 822-828.

Duan, J., Jiang, W., Cheng, Z., Heikkila, J.J., Glick, B.R., 2013. The complete genome sequence of the plant growth-promoting bacterium Pseudomonas sp. UW4. PLoS ONE 8, 58640.

Duffus, J.H., 2002. Heavy metals— a meaningless term? Pure Applied Chemistry 74, 793–807.

Duman, F., Ozturk, F., 2010. Nickel accumulation and its effect on biomass, protein content and antioxidative enzymes in roots and leaves of watercress (*Nasturtium officinale R. Br*.). Journal of Environmental Sciences 22, 526-532.

Duqucne, L., Vandenhove, H., Tack, F., Meers, E., Baeten, J., Wannijn, J., 2009. Enhanced phytoextraction of uranium and selected heavy metals by Indian mustard and ryegrass using biodegradable soil amendments. Science of the Total Environment 407, 1496-1505.

Durán, N., Durán, M., de Jesus M.B., Seabra, A.B., Fávaro, W.J., Nakazato, G., 2016. Silver nanoparticles: a new view on mechanistic aspects on antimicrobial activity. Nanomedicine; Nanotechnology Biology and Medicine 12, 789–799.

Durham, P.J., 1988. Theory of XANES. In X-ray absorption: principles, applications and Techniques of EXAFS, SEXAFS and XANES. John Wiley. pp. 53-84.

Dushenkov, V., Kumar, P.B.A.N., Motto, H., Raskin, I., 1995. The use of plants to remove heavy metals from aqueous streams. Environmetal Science and Technology 29, 1239-1245.

Dushenkov, S., Vasudev, D.D., Kapulnik, Y., Gleba, D., Fleisher, D., Ting, K.C., Ensley, B., 1997. Removal of uranium from water using terrestrial plants. Environmental Science and Technology 31, 3468-3474.

Dushenkov, S., Kapulnik, Y., 2000. Phytofiltration of metals. In phytoremediation of toxic metals: using plants to clean up the environment. New York: John Wiley and Sons. pp. 89-106.

EA (Environment Agency in England and Wales)., 2009a. Updated Technical Background to the CLEA Model. Science Report SC050021/SR3, Bristol, UK.

EA (Environment Agency in England and Wales)., 2009b. CLEA Software (Version 1.04) Handbook. Science Report SC050021/SR4, Bristol, UK.

Eapen, S., Suseelan, K.N., Tivarekar, S., Kotwal, S.A., Mitra, R., 2003. Potential for rhizofiltration of uranium using hairy root cultures of *Brassica juncea* and *Chenopodium amaranticolor*. Environmental Research 91, 127-133.

Ebbs, S.D., Lasat, M.M., Brady, D.J., Cornish, J., Gordon, R., Kochian, L.V., 1997. Heavy metals in the environment: Phytoextraction of cadmium and zinc from a contaminated soil. Journal of Environmental Quality 26, 1424–1430.

Ebbs, S.D., Kochian, L.V., 1997. Toxicity of zinc and copper to *Brassica* species: Implications for phytoremediation. Environmental Quality 26, 776-781.

Ebbs, S.D., Kochian, L.V., 1998. Phytoextraction of zinc by Oat (*Avena sativa*), Barley (*Hordeum vulgare*), and Indian mustard (*Brassica juncea*). Environmental Science and Technology 32, 802-806.

Ebbs, S., Uchil, S., 2008. Cadmium and zinc induced chlorosis in Indian mustard (*Brassica juncea (L.) Czern*) involves preferential loss of chlorophyll b. Photosynthetica 46, 49-55.

Ebbs, S.D., Bradfield, S.J., Kumar, P., White, J.C., Musante, C., Ma, X., 2016. Accumulation of zinc, copper, or cerium in carrot (Daucus carota) exposed to metal oxide nanoparticles and metal ions. Environmental Science: Nano 3, 114-126.

El Kassas, H.I., Sharaf, M.S., Mousa, I.A., Niazy, M.M., 2003. Phytoremediation of zinc and copper contaminated soils using different hyperaccumulating plants. Journal of Environmental Science 7, 3.

Elder, J.F., 1989. Metal biogeochemistry in surface-water systems. A review of principles and concepts: U.S. Geological Survey Circular 1013, 43.

Elekes, C.C., 2014. Eco-technological solutions for the remediation of polluted soil and heavy Metal recovery. Environmental risk assessment of soil contamination. InTech, Rijeka. pp. 309-335.

Elliot, H.A., Liberati, M.R., Huang, C.P., 1986. Competitive sorption of heavy metals by soils. Journal of Environmental Quality 15, 214-218.

ENRHES., 2010. Engineered Nanoparticles: Review of Health and Environmental Safety, E.U.

Environment Agency., 2002. Assessment of risks to human health from land contamination: an overview of the development of soil guideline values and related research DEFRA, Bristol, UK.

Environment Agency., 2004. The state of soils in England and Wales. Bristol: Environment Agency.

Eren, E., Arguello, J.M., 2004. Arabidopsis *HMA2*, a divalent heavy metal-transporting PIB-type ATPase, is involved in cytoplasmic Zn^{2+} homeostasis. Plant Physiology 136, 3712–3723.

Erten-Unal, M., Wixsonm, B.G., Gale, N., Pitt, J.L., 1998. Evaluation of toxicity, bioavailability and speciation of lead, zinc and cadmium in mine/mill wastewaters. Chemical Speciation and Bioavailability 10, 37-46.

Essington, M.E., 2004. Soil and water chemistry: an integrative approach. CRC Press, Boca Raton. pp. 1-534.

European Commission, 2013. Science for Environment Policy In-depth Report: Soil Contamination: Impacts on Human Health. Report produced for the European Commission DG Environment, September, 2013. Science Communication Unit, University of the West of England, Bristol. Available at: http://ec.europa.eu/science-environment-policy. [Accessed 5 April 2016].

Evans, L.J., 1989. Chemistry of metal retention by soils. Environmental Science and Technology 23, 1046–1056.

Fageria, N.K., Stone, L.F., 2006. Physical, chemical, and biological changes in the rhizosphere and nutrient availability. Journal of Plant Nutrition 29, 1327–1356.

Faisal, M., Saquib, Q., Alatar, A.A., Al-Khedhairy, A.A., Hegazy, A.K., Musarrat, J., 2013. Phytotoxic hazards of NiO-nanoparticles in tomato: a study on mechanism of cell death. Journal of Hazardous Materials 250, 318–332.

Farrell, R.E., Germida, J.J., Huang, P.M., 1993. Effects of chemical speciation in growth media on the toxicity of mercury (II). Applied and Environmental Microbiology 59, 1507-1514.

Feigl, G., Lehotai, N., Molnár, Á., Ördög, A., Rodríguez-Ruiz, M., Palma, J.M., Corpas, F.J., Erdei, L., Kolbert, Z., 2015. Zinc induces distinct changes in the metabolism of reactive oxygen and nitrogen species (ROS and RNS) in the roots of two *Brassica* species with different sensitivity to zinc stress. Annals of Botany 116, 613-625.

Fekiacova, Z., Cornu, S., Pichat, S., 2015. Tracing contamination sources in soils with Cu and Zn isotopic ratios. Science of the Total Environment 517, 96-105.

Feng, Y., Cui, X., He, S., Dong, G., Chen, M., Wang, J., Lin, X., 2013. The role of metal nanoparticles in influencing Arbuscular mycorrhizal fungi effects on plant growth. Environmental Science and Technology 47, 9496−9504.

Fernandez, E., Jimenez, R., Lallena, A.M., Aguilar, J., 2004. Evaluation of the BCR sequential extraction procedure applied for two unpolluted Spanish soils. Environmental Pollution 131, 355-364.

Filgueiras, A.V., Lavilla, I., Bendicho, C., 2002. Chemical sequential extraction for metal Partitioning in environmental solid samples. Journal of Environment and Monitoring 4, 823-857.

Fitter, A.H., 1987. An architectural approach to the comparative ecology of plant root systems. New Phytologist 106, 61–77.

Fitz, W.J., Wenzel, W.W., 2002. Arsenic transformations in the soil–rhizosphere–plant system: fundamentals and potential application to phytoremediation. Journal of Biotechnology 99, 259-278.

Flowers, R.S., Ordal, Z.J., 1979. Current methods to detect stressed *staphylococci*. Journal of Food Protection 42, 362-367.

Fonseca, E.C., Martin, H., 1986. The selective extraction of Pb and Zn in selected mineral and soil samples, application in geochemical exploration (Portugal). Journal of Geochemical Exploration 26, 231-248.

Fotovat, A., Naidu, R., 1998. Changes in composition of soil aqueous phase influence chemistry of indigenous heavy metals in alkaline sodic and acidic soils. Geoderma 84, 213-234.

Fowke, L.C., 1995. Transmission and scanning electron microscopy for plant protoplasts, cultured cells and tissues. Berlin, Springer-Verlag. pp. 229-238.

Fowler, T.A., Crundwell, F.K., 1998. Leaching of zinc sulfide by *Thiobacillus ferrooxidans*: experiments with a controlled redox potential indicate no direct bacterial mechanism. Applied and Environmental Microbiology 64, 3570-3575.

Foy, C.D., Chaney, R.L., White, M.C., 1978. The physiology of metal toxicity in plants. Annual Review of Plant Physiology 29, 511-566.

Franklin, N.M., Rogers, N.J., Apte, S.C., Batley, G.E., Gadd, G.E., Casey, P.S., 2007. Comparative toxicity of nanoparticulate ZnO, bulk ZnO, and $ZnCl_2$ to a freshwater microalga (*Pseudokirchneriella subcapitata*): The importance of particle solubility. Environmental Science and Technology 41, 8484–8490.

Friehs, E., AlSalka, Y., Jonczyk, R., Lavrentieva, A., Jochums, A., Walter, J.G., Stahl, F., Scheper, T., Bahnemann, D., 2016. Toxicity, phototoxicity and biocidal activity of nanoparticles employed in photocatalysis. Journal of Photochemistry and Photobiology C: Photochemistry Reviews 29, 1-28.

Fuentes, A., Llorens, M., Saez, J., Aguilar, M.I., Ortuno, J., 2004. Phytotoxicity and heavy metals speciation of stabilized sewage sludges. Journal of Hazardous Materials 108, 161-169.

Fultz, B., Howe, J.M., 2012. Transmission electron microscopy and diffractometry of materials. Science and Business Media, Springer. pp. 59-115.

Gadd, G. M., 1992. Metals and microorganisms: a problem of definition. FEMS Microbiology Letters 100, 197–204.

Gadd, G.M., 2010. Metals, minerals and microbes: geomicrobiology and bioremediation.

Galende, M.A., Becerril, J.M., Barrutia, O., Artetxe, U., Garbisu, C., Hernández, A., 2014. Field assessment of the effectiveness of organic amendments for aided phytostabilization of a Pb–Zn contaminated mine soil. Journal of Geochemical Exploration 145, 181-189.

Gamalero, E., Glick, B.R., 2012. Plant growth-promoting bacteria and metal phytoremediation. In Phytotechnologies. Anjum, N.A., Pereira, M.E., Ahmad, I., Duarte, A.C., Umar, S., Khan, N.A. Eds. Taylor and Francis, Boca Raton, USA. pp. 359–374.

Gambrell, R.P., Wiesepape, J.B., Patrick, W.H., Duff, M.C., 1991. The effects of pH, redox, and salinity on metal release from a contaminated sediment. Water Air and Soil Pollution 57, 359-367.

Ganguly, S., Das, S., Dastidar, S.G., 2014. Study of antimicrobial effects of the anticancer drug oxaliplatin and its interaction with synthesized ZnS nanoparticles. International Journal of Pharmacy and Therapeutics 5, 230-234.

Gardea-Torresdey, J.L., Peralta-Videa, J.R., De La Rosa, G., Parsons, J.G., 2005. Phytoremediation of heavy metals and study of the metal coordination by X-ray absorption spectroscopy. Coordination Chemistry Reviews 249, 1797-1810.

Gardea-Torredsey, J.L., Rico, C.M., White, J.C., 2014. Trophic transfer, transformation and impact of engineered nanomaterials in the terrestrial environments. Environmental Science and Technology 48, 2526-2540.

Garner, K.L., Keller, A. A., 2014. Emerging patterns for engineered nanomaterials in the environment: a review of fate and toxicity studies. Journal of Nanoparticle Research 16, 2503.

Gaur, A., Shrivastava, B.D., Joshi, S.K., 2009. Copper K-edge XANES of Cu (I) and Cu (II) oxide mixtures. In Journal of Physics: Conference Series 190, 1.

Gaw, S., 2009. Options for assessing the bioavailability of metals to soil dwelling organisms. Chemistry in New Zealand 73, 150–155.

Ge, Y., Schimel, J.P., Holden, P.A., 2011. Evidence for negative effects of TiO_2 and ZnO nanoparticles on soil bacterial communities. Environmental Science and Technology 45, 1659-1664.

Gerhardt, K.E., Huang, X., Glick, B.R., Greenberg, B.M., 2009. Phytoremediation and rhizoremediation of organic soil contaminants: potential and challenges. Plant Science 176, 20-30.

Germaine, K.J., Keogh, E., Ryan, D., Dowling, D.N., 2009. Bacterial endophyte-mediated naphthalene phytoprotection and phytoremediation. FEMS Microbiology Letters 296, 226–234.

Ghasemzadeh, G., Momenpour, M., Omidi, F., Hosseini, M.R., Ahani, M., Barzegari, A., 2014. Applications of nanomaterials in water treatment and environmental remediation. Frontiers of Environmental Science and Engineering 8, 471-482.

Ghosh, M., Singh, S.P., 2005. A review on phytoremediation of heavy metals and utilization of it's by products. Applied Ecology and Environmental Research 3, 1-18.

Gigliotti, G., Businelli, D., Giusquiani, P.L., 1996. Trace metals and distribution in corn plants grown on a 6-year urban waste compost amended soil. Agriculture, Ecosystem and Environment 58, 199-206.

Gill, R.A., Zang, L., Ali, B., Farooq, M.A., Cui, P., Yang, S., Ali, S., Zhou, W., 2015. Chromium-induced physio-chemical and ultrastructural changes in four cultivars of *Brassica napus L*. Chemosphere 120, 154–164.

Giordani, C., Cecchi, S., Zanchi, C., 2005. Phytoremediation of soil polluted by nickel using agricultural crops. Environmental Management 36, 675-681.

Gladkova, M.M., Terekhova, V.A., 2013. Engineered nanomaterials in soil: Sources of entry and migration pathways. Moscow University Soil Science Bulletin 68, 129-134.

Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology 41, 109-117.

Glick, B.R., Karaturovic, D.M., Newell, P.C., 1995. A novel procedure for rapid isolation of plant growth promoting pseudomonads. Canadian Journal of Microbiology 41, 533-536.

Glick, B.R., Penrose, D.M., Li, J., 1998. A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. Journal of Theoretical Biology 190, 63-68.

Glick, B.R., Patten, C.L., Holguin, G., Penrose, D.M., 1999. Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London, United Kingdom. pp. 267

Glick, B.R., 2004. Bacterial ACC deaminase and the alleviation of plant stress. Advances in Applied Microbiology 56, 291-312.

Glick, B.R., Cheng, Z., Czarny, J., Duan, J., 2007. Promotion of plant growth by ACC deaminase-producing soil bacteria. European Journal of Plant Pathology 119, 329–39.

Glick, B.R., 2010. Using soil bacteria to facilitate phytoremediation. Biotechnology Advances 28, 367–374.

Glick, B.R., 2012. Plant growth-promoting bacteria: mechanisms and applications. Scientifica 2012.

Glover, R.D., Miller, J.M., Hutchison, J.E., 2011. Generation of metal nanoparticles from silver and copper objects: Nanoparticle dynamics on surfaces and potential sources of nanoparticles in the environment. ACS Nano 5, 8950-8957.

Glusker, J.P., 1991. Structural aspects of metal liganding to functional groups in proteins. Advances in Protein Chemistry 42, 1-76.

Gnandi, K., Tobschall, H., 2002. Heavy metals distribution of soils around mining sites of cadmium-rich marine sedimentary phosphorites of Kpogame and Hahotoe (southern Togo). Environmental Geology 41, 593-600.

Goix, S., Uzu, G., Oliva, P., Barraza, F., Calas, A., Castet, S., Point, D., Masbou, J., Duprey, J.L., Huayta, C., Chincheros, J., 2016. Metal concentration and bioaccessibility in different particle sizes of dust and aerosols to refine metal exposure assessment. Journal of Hazardous Materials 317, 552–562.

Gomes, M.P., Duarte, D.M., Carneiro, M.M.L.C., Barreto, L.C., Carvalho M., Soares, A.M., Guilherme, L.R.G., Garcia, Q.S., 2013. Zinc tolerance modulation in *Myracrodruon urundeuva* plants. Plant Physiology and Biochemistry 67, 1–6.

Gomes, M.A., Hauser-Davis, R.A., de Souza, A.N., Vitória, A.P., 2016. Metal phytoremediation: General strategies, genetically modified plants and applications in metal nanoparticle contamination. Ecotoxicology and Environmental Safety 134, 133-147.

Gräfe, M., Donner, E., Collins, R.N., Lombi, E., 2014. Speciation of metal (loid) s in environmental samples by X-ray absorption spectroscopy: a critical review. Analytica Chimica Acta 822, 1-22.

Gramlich, A., Tandy, S., Frossard, E., Eikenberg, J., Schulin, R., 2013. Availability of zinc and the ligands citrate and histidine to wheat: does uptake of entire complexes play a role? Journal of Agricultural and Food Chemistry 61, 10409-10417.

Grandlic, C.J., Palmer, M.W., Maier, R.M., 2009. Optimization of plant growth – promoting bacteria- assisted phytormediation of mine tailings. Soil Biology and Biochemistry 41, 1734-1740.

Gray, E.J., Smith, D.I., 2004. Intracellular and extracellular PGPR. Soil Biology and Biochemistry 5, 1-18.

Grewal, H.S., Graham, R.D., 1997. Seed zinc content influences early vegetative growth and zinc uptake in oilseed rape (*Brassica napus* and *Brassica juncea*) genotypes on zinc-deficient soil. Plant and Soil 192, 191-197.

Griffitt, R.J., Weil, R., Hyndman, K.A., Denslow, N.D., Powers, K., Taylor, D., Barber, D.S., 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). Environment Science and Technology 41, 8178–8186.

Grill, E., Winnacker, E.L., Zenk, M.H., 1985. Phytochelatins: the principal heavy-metal complexing peptides of higher plants. Science 230, 674-676.

Gu, Y.G., Lin, Q., Gao, Y.P., 2016. Metals in exposed-lawn soils from 18 urban parks and its human health implications in southern China's largest city, Guangzhou. Journal of Cleaner Production 115, 122-129.

Guar, A., Adholeya, A., 2004. Prospects of abuscular mycorrhizal fungi in pytoremediation of heavy metal contaminated soils. Current Science 86, 528-534.

Guney, M., Zagury, G.J., 2016. Bioaccessibility and other key parameters in assessing oral exposure to PAH-contaminated soils and dust: A critical review. Human and Ecological Risk Assessment 22, 1396–1417.

Guo, K., Liu, Y.F., Zeng, C., Chen, Y.Y., Wei X.J., 2014. Global research on soil contamination from 1999 to 2012: A bibliometric analysis, Acta Agriculturae Scandinavica, Section B — Soil and Plant Science 64, 377-391.

Gupta, C., Dubey, R., Maheshwari, D., 2002. Plant growth enhancement and suppression of *Macrophomina phaseolina* causing charcoal rot of peanut by *fluorescent Pseudomonas*. Biology and Fertility of Soils 35, 399-405.

Guzman, P., Fernandez, V., Khayet, M., Garcia, M.L., Fernandez, A., Gil, L., 2014. Ultrastructure of plant leaf cuticles in relation to sample preparation as observed by transmission electron microscopy. The Scientific World Journal 963921, 9.

Hafeez, B., Khanif, Y.M., Saleem, M., 2013. Role of zinc in plant nutrition- A review American Journal of Experimental Agriculture 3, 374-391.

Haghighi, B.J., Alizadeh, O., Firoozabadi, A.H., 2011. The role of plant growth promoting rhizobacteria (PGPB) in sustainable agriculture. Advances in Environmental Biology 5, 3079-3083.

Hall, J.L., 2002. Cellular mechanisms for havy metal detoxification and tolerance. Journal of Experimental Botany 53, 1-11.

Hammer, D., Keller, C., 2002. Changes in the rhizosphere of metal-accumulating plants evidenced by chemical extractants. Journal of Environmental Quality 31, 1561-1569.

Hamon, R., Mclaughlin, M., 2003. Food crop edibility on the OK Tedi/ Fly River flood plain. Report for OK Tedi Mining Ltd. CSIRO Australian center for environmental contaminant research, a one CSIRO centre. Available at http://www.oktedi.com/attachments/246_030815_Food%20Crop%20Edibility_Hammon%20&%20McLaughlin_CSIRO_FINAL.pdf. [Accessed 3 April 2012].

Hamon, R.E., Holm, P.E., Lorenz, S.E., McGrath, S.P., Christensen, T.H., 1999. Metal uptake by plants from sludge-amended soils: caution is required in the plateau interpretation. Plant and Soil 216, 53-64.

Hamon, R.E., Wundke, J., McLaughlin, M.J., Naidu, R., 1997. Availability of zinc and cadmium to different plant species. Australian Journal of Soil Research 35, 1267-1277.

Hani, H., 1996. Soil analysis as a tool to predict effects on the environment. Communications in Soil Science and Plant Analysis 27, 289-306.

Harmsen, J., 2007. Measuring bioavailability: From a scientific approach to standard methods. Journal of Environmental Quality 36, 1420-1428.

Harter, R.D., 1983. Effect of soil pH on adsorption of lead, copper, zinc, and nickel. Soil Science Society of American Journal 47, 47–51.

Hayat, R., Ali, S., Siddique, M.T., Chatha, T.H., 2008a. Biological nitrogenfixation of summer legumes and their residual effects on subsequent rainfed wheat yield. Pakistan Journal of Botany 40, 711-722

Hayat, R., Ali, S., Siddique, M.T., Ijaz, S.S., Chatha, T.H., 2008b. Estimation of N - fixation of mung bean and mash bean through xylem uriedetechnique under rainfed conditions. Pakistain Journal of Botany 40, 723-734.

Hayat, R., Safdar, A., Amara, U., Khalid, R., Ahmed, I., 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. Annals of Microbiology 60, 579–598.

Hayes, S.M., White, S.A., Thompson, T.L., Maier, R.M., Chorover, J., 2009. Changes in lead and zinc lability during weathering-induced acidification of desert mine tailings: Coupling chemical and micro-scale analyses. Applied Geochemistry 24, 2234.

He, Q., Ren, Y., Mohamed, I., Ali, M., Hassan, W., Zeng, F., 2013. Assessment of trace and heavy metal distribution by four sequential extraction procedures in a contaminated soil. Soil and Water Research 8, 71–76.

Hegde, K., Brar, S.K., Verma, M., Surampalli, R.Y., 2016. Current understandings of toxicity, risks and regulations of engineered nanoparticles with respect to environmental microorganisms. Nanotechnology for Environmental Engineering 1, 5.

Heinlaan, M., Ivask, A., Blinova, I., Dubourguier, H., Kahru, A., 2008. Toxicity of nanosizes and bulk ZnO, CuO, and TiO_2 to bacteria *Vilbrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. Chemosphere 71, 1308-1316.

Hernandez-Viezcas, J.A., Castillo-Michel, H., Servin, A.D., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2011. Spectroscopic verification of zinc absorption and distribution in the desert plant *Prosopis juliflora-velutina* (velvet mesquite) treated with ZnO nanoparticles. Chemical Engineering Journal 170, 346-352.

Hernandez-Viezcas, J.A., Castillo-Michel, H., Andrews, J.C., Cotte, M., Rico, C., Peralta-Videa, J.R., Ge, Y., Priester, J.H., Holden, P.A. and Gardea-Torresdey, J.L., 2013. In situ synchrotron X-ray fluorescence mapping and speciation of CeO_2 and ZnO nanoparticles in soil cultivated soybean (Glycine max). ACS Nano 7, 1415-1423.

Hettiarachchi, G., Pierzynski, G., Ransom, M., 2001. In situ stabilization of soil lead using phosphorus. Journal of Environmental Quality 30, 1214-1221.

Hinsinger, P., Bengough, A.G., Vetterlein, D., Young, I.M., 2009. Rhizosphere: biophysics, biogeochemistry and ecological relevance. Plant and Soil 321, 117-152.

Hinsinger, P., Courchesne, F., 2008. Biogeochemistry of metals and metalloids at the soil–root interface. Biophysico- chemical processes of heavy metals and metalloids in soil environments. John Wiley and Sons. pp. 267–310.

Hinsinger, P., Gobran, G.R., Gregory, P.J., Wenzel, W.W., 2005. Rhizosphere geometry and heterogeneity arising from root-mediated physical and chemical processes. New Phytologist 168, 293-303.

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method of growing plants without soil. California Agricultural Experimental Station Circular 347, 1-32.

Hochella, M.F., Spencer, M.J., Jones, K.L., 2015. Nanotechnology: nature's gift or scientists' brainchild? Environmental Science: Nano 2, 114-119.

Hoflich, G., 2000. Colonization and growth promotion of non-legumes by Rhizobium bacteria. Micobial biosystems: new prontiers. Proceedings of the 8th international symposium on microbial ecology. Atlantic Canada Society, Microbial Ecology, Halifax, Canada. pp. 827-830.

Homayoun, H., 2011. Effect of NaCl salinity on wheat (*Triticum aestivum L*.) cultivars at germination stage. Advances in Environmental Biology 5, 1716-1720.

Hong, F., Zhou, J., Liu, C., Yang, F., Wu, C., Zheng, L., Yang, P., 2005. Effect of nano-TiO_2 on photochemical reaction of chloroplasts of spinach. Biological Trace Element Research 105, 269-279.

Hossain, M.O., Piyatida, P., Teixeira da Silva, J.A., Fujita, M., 2012. Molecular mechanism of heavy metal toxicity and tolernace in plants: central glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. Journal of Botany 37.

Hou, D., Al-Tabbaa, A., 2014. Sustainability: A new imperative in contaminated land remediation. Environmental Science and Policy 39, 25-34.

Hough, R.L., Breward, N., Young, S.D., Crout, N.M., Tye, A.M., Moir, A.M., Thornton, I., 2004. Assessing potential risk of heavy metal exposure from consumption of home-produced vegetables by urban populations. Environmental Health Perspectives 112, 215.

Howe, J.M., Fultz, B., Miao, S., 2012. Transmission electron microscopy. Characterization of Material 1-46.

Hu, P.J., Qui, R.L., Senthilkumar, P., Jiang, D., Chen, Z.W., Tang, Y.T., Liu, F.J., 2009. Tolerance, accumulation and distribution of zinc and cadium in hyperacumulator *Potentilla griffithii*. Environmental and Experimental Botany 66, 317-325.

Huang, J.W., Chen, J., Berti, W.R., Cunningham, S.D., 1997. Phytoremediation of lead contaminated soils-role of synthetic chelates in lead phytoextraction. Environmental Science and Technology 31, 800-806.

Huang, X.D., El-Alawi, Y., Gurska, J., Glick, B.R., Greenberg, B.M., 2004. A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environmental Pollution 130, 465–476.

Hussain, D., Haydon, M.J., Wang, Y., Wong, E., Sherson, S.M., Young, J., Camakaris, J., Harper J.F., Cobbett C.S., 2004. P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. The Plant Cell 16, 1327–1339.

Ianni, C., Bignasca, A., Magi, E., Rivaro, P., 2010. Metal bioavailability in marine sediments measured by chemical extraction and enzymatic mobilization. Microchemical Journal 96, 308-316.

Ianni, C., Bignasca, A., Calace, N., Rivaro, P., Magi, E., 2014. Bioaccessibility of metals in soils: comparison between chemical extractions and in vitro tests. Chemistry and Ecology 30, 541-554.

Isaure, M.P., Laboudigue, A., Manceau, A., Sarret, G., Tiffreau, C., Trocellier, P., Lamble, G., Hazemann, J.L., Chateigner, D., 2002. Quantitative Zn speciation in a contaminated dredged sediment by μ-PIXE, μ-SXRF, EXAFS spectroscopy and principal component analysis. Geochimica et Cosmochimica Acta 66, 1549-1567.

Isaure, M.P., Fayard, B., Sarret, G., Pairis, S., Bourguignon, J., 2006. Localization and chemical forms of cadmium in plant samples by combining analytical electron microscopy and X-ray spectromicroscopy. Spectrochimica Acta Part B: Atomic Spectroscopy 61, 1242-1252.

Islam, E., Yang, X.E., He, Z.L., Mahmood, Q., 2007. Assessing potential dietary toxicity of heavy metals in selected vegetables and food crops. Journal of Zhejiang University Science B 8, 1-13.

ISO, 1995. Soil quality. Extraction of trace elements soluble in aqua regia. ISO 11466.

Israr, M., Sahi, S.V., Jain, J., 2006. Cadmium accumulation and antioxidative responses in the *Sebania Drummondii callus*. Archives of Environmental Contamination Toxicology 50, 121-127.

ISTA., 2008. International Rules for Seed Testing (ISTA) 2008. International Seed Testing Association Germination test. pp. 1-57.

Jaffre, T., Brooks, R.R., Lee, J., Reeves, R.D., 1976. *Serbatia acumin* a nickel-accumulating plant from New Caledonia. Science 193, 579-580.

Jain, C.K., Ram, D., 1997. Adsorption of metal ions on bed sediments. Hydrological Sciences 42, 713-723.

Jain, R., Srivastava, S., Solomon, S., Shrivastava, A.K., Chandra, A., 2010. Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane (*Saccharum spp*.). Acta Physiologiae Plantarum 32, 979-986.

Janczarek, M., Rachwal, K., Cieśla, J., Ginalska, G., Bieganowski, A., 2015. Production of exopolysaccharide by *Rhizobium leguminosarum bv. trifolii* and its role in bacterial attachment and surface properties. Plant Soil 388, 211–227.

Jeffery, J.J., Uren, N.C., 1983. Copper and zinc species in the soil solution and the effects of soil pH. Soil Research 21, 479-488.

Jez, J.M., Dey, S., 2013. The cysteine regulatory complex from plants and microbes: what was old is new again. Current Opinion in Structural Biology 23, 302-310.

Jez, J.M., Ravilious, G.E., Herrmann, J., 2016. Structural biology and regulation of the plant sulfation pathway. Chemico-Biological Interaction 1-8. doi:10.1016/j.cbi.2016.02.017.

Jiang, G., Zeng, J., He, Y., 2014. Analysis of quantitative trait loci affecting chlorophyll content of rice leaves in a double haploid population and two backcross populations. Gene 536, 287–295.

Jin, X.F., Lui, D., 2009. Effects of zinc on root morphology and antioxidant adaptations of cadmium- treated *Sedum alfredii H*. Journal of Plant Nutrition 32, 1642-1656.

Jing, Y., He, Z., Yang, X., 2007. Role of soil rhizobacteria in phytoremediation of heavy metal contaminated soils. Journal of Zhejiang University - Science B 8, 192–207.

Johnson, A., Singhal, N., 2011. A review of amendment-enhanced phytoextraction of soil contaminants. Environmental Research Journal 5, 371- 425.

Jones, D.L., 1998. Organic acids in the rhizosphere - a critical review. Plant Soil 205, 25-44.

Jones, N., Ray, B., Ranjit, K.T., Manna, A.C., 2008. Antibacterial activity of ZnO nanoparticle suspensions on a broad spectrum of microorganisms. FEMS Microbiology Letters 279, 71-76.

Ju-Nam, Y., Lead, J.R., 2008. Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications. Science of the Total Environment 400, 396-414.

Kabata-Pendias, A., Pendias, H., 1984. Trace elements in soils and plants. CRC Press, Boca Raton, USA.

Kabata-Pendias, A., Pendias H., 2001. Trace elements in soils and plants, CRC Press, Boca.

Kabata-Pendias, A., 2004. Soil–plant transfer of trace elements an environmental issue. Geoderma 122, 143–149.

Kahru, A., Dubourguier, H.C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269, 105- 119.

Kaiser, C., Kilburn, M.R., Clode, P.L., Fuchslueger, L., Koranda, M., Cliff, J.B., Solaiman, Z.M., Murphy, D.V., 2015. Exploring the transfer of recent plant photosynthates to soil microbes: mycorrhizal pathway vs direct root exudation. New Phytologist 205, 1537-1551.

Kalve, S., Sarangi, B.K., Pandey, R.A., Chakrabarti, T., 2011. Arsenic and chromium hyperaccumulation by an ecotype of Pteris vittata – prospective for phytoextraction from contaminated water and soil. Current Science 100, 888–894.

Karn, B., Kuiken, T., Otto, M., 2009. Nanotechnology and in situ remediation: A review of the benefits and potential risks. Environmental Health Perspective 117, 1823-1831.

Kasim, W.A., 2007. Physiological consequences of structural and ultra-structural changes induced by Zn stress in *Phaseolus vulgaris. I.* growth and photosynthetic apparatus. International Journal of Botany 3, 15-22.

Katyal, J.C., Randhawa, N.S., 1983. Rome: food and agriculture organization of the United Nations. Micronutrients FAO Fertilizer and Plant Nutrition Bulletin 7.

Kawai, J., 2000. Adsorption technique in X-ray spectrometry. Encyclopedia of Analytical Chemistry R.A. Meyers. John Wiley and Sons, Chichester. pp. 13288-13315.

Kaya, C., Higgs, D., Burton, A., 2000. Plant growth, phosphorus nutrition and acid phosphatase enzyme activity in three tomato cultivers grown hydroponically at different zinc concentrations. Journal of Plant Nutrition 23, 569-579.

Keller, A.A., McFerran, S., Lazareva, A., Suh, S., 2013. Global life cycle releases of engineered nanomaterials. Journal of Nanoparticles Research 15, 1–17.

Khan, A.G., 2005. Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. Journal of Trace Elements in Medicine and Biology 18, 355–364.

Khan, F.I., Husain. T., Hejazi, R., 2004. An overview and analysis of site remediation technologies. Journal of Environmental Management 71, 95–122.

Khan, M.S., Zaidi, A., Wani, P.A., Oves, M., 2009. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environmental Chemistry Letters 7, 1–19.

Khan, S., Cao, Q., Zheng, Y.M., Huang, Y.Z., Zhu, Y.G., 2008. Health risks of heavy metals in contaminated soils and food crops irrigated with wastewater in Beijing, China. Environmental Pollution 152, 686–692.

Khanna, P., Ong, C., Bay, B. H., Baeg, G. H., 2015. Nanotoxicity: an interplay of oxidative stress, inflammation and cell death. Nanomaterials 5, 1163-1180.

Khudsar, T., Igbal, M., Sairam, R., 2004. Zinc induced changes in morpho-physiological and biochemical parameters in Artemisia. Biologia Plantarum 48, 255-260.

Kidd, P.S., Proctor, J., 2001. Why plants grow poorly on very acid soils: are ecologists missing the obvious? Journal of Experimental Botany 52, 791-799.

Kidd, P., Barceló, J., Bernal, M.P., Navari-Izzo, F., Poschenrieder, C., Shilev, S., Clemente, R., Monterroso, C., 2009. Trace element behaviour at the root–soil interface: implications in phytoremediation. Environmental and Experimental Botany 67, 243-259.

Kim, K.R., Owens, G., Kwon, S.L., 2010. Influence of Indian mustard (*Brassica juncea*) on rhizosphere soil solution chemistry in long-term contaminated soils: a rhizobox study. Journal of Environmental Sciences 22, 98-105.

Kim, R.Y., Yoon, J.K., Kim, T.S., Yang, J.E., Owens, G., Kim, K.R., 2015. Bioavailability of heavy metals in soils: definitions and practical implementation—a critical review. Environmental Geochemistry and Health 37, 1041-1061.

Kim, S., Lee, S., Lee, I., 2012. Alteration of phytotoxicity and oxidant stress potential by metal oxide nanoparticles in Cucumis sativus. Water, Air, and Soil Pollution 223, 2799-2806.

Kim, S., Sin, H., Lee, S., Lee, I., 2013. Influence of metal oxide particles on soil enzyme activity and bioaccumulation of two plants. Journal of Microbiology Biotechnology 23, 1279–1286.

Kinraide, T.B., 2009. Improved scales for metal ion softness and toxicity. Environmental Toxicology and Chemistry 28, 525-533.

Kjeldahl, J., 1883. A new method for the determination of nitrogen in organic matter. Analytical Chemistry 22, 366.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mehendra, S., Mclaughlin, M.J., lead, J.R., 2008. Nanomaterial in the environment; behaviour, fate, bioavailability and effects. Environmental Toxicology and Chemistry 27, 1825-1851.

Kloepper, J.W., Schroth, M.N., 1978. Plant growth- promoting rhizobacteria on radishes. In Proceedings of the 4th international conference on plant pathogenic bacteria. pp. 879-882.

Kloepper, J.W., Zablolowicz, R.M., Tipping, E.M., Lifshitz, R., 1991. Plant growth promotion mediated by bacteria rhizosphere colonizer. The rhizosphere and plant growth. Kluwer Academic Publishers, Netherlands. pp. 315-326.

Kolodynska, D., 2013. Application of a new generation of complexing agents in removal of heavy metal ions from different wastes. Environmental Science and Pollution Research International 20, 5939–5949.

Koopmans, G.F., Romkens, P.M., Fokkema, M.J., Song, J., Luo, Y.M., Japenga, J., Zhao, F.J., 2008. Feasibility of phytoextraction to remediate cadmium and zinc contaminated soils. Environmental Pollution 156, 905-914.

Kopittke, P.M., Menzies, N.W., de Jonge, M.D., Mckenna, B.A., Donner, E., Webb, R.I., Patterson, D.J., Howard, D.I., Ryan, C.G., Glover, C.J., 2011. In situ distribution and speciation of toxic copper, nickel, and zinc in hydrated roots of cowpea. Plant Physiologist 156, 663-673.

Koppolua, L., Agblover, F.A., Clements, L.D., 2003. Pyrolysis as a technique for separating heavy metals from hyperaccumulators. Part II: Lab-scale pyrolysis of synthetic hyperaccumulator biomass. Biomass and Bioenergy 25, 651–663.

Kopponen, P., Utriainen, M., Lukkari, K., Suntioinen, S., Karenlampi, L., Karenlampi, S., 2001. Clonal differences in copper and zinc tolerance of birch in metal-supplemented soils. Environmental Pollution 112, 89-97.

Koptsik, G.N., 2014. Problems and prospects concerning the phytoremediation of heavy metal polluted soils: a review. Eurasian Soil Science 47, 923–939.

Kos, B., Greman, H., Lestan, D., 2003. Phytoextraction of lead, zinc and cadmium from soil by selected plants. Plant Soil and Environment 49, 548-553.

Kouhi, S.M.M., Lahouti, M., Ganjeali, A., Entezari, M.H., 2015. Long-term exposure of rapeseed (Brassica napus L.) to ZnO nanoparticles: anatomical and ultrastructural responses. Environmental Science and Pollution Research 22, 10733-10743.

Kourkoutis, L.F., Plitzko, J.M., Baumeister, W., 2012. Electron microscopy of biological materials at nanometer scale. Annual Review of Material Research 42, 33-58.

Kramer, U., Cotter-Howells, J.D., Charnock, J.M., Baker A.J.M., Smith, A.C., 1996. Free histidine as a metal chelator in plants that accumulate nickel. Nature 379, 635–638.

Krämer, U., Pickering, I.J., Prince, R.C., Raskin, I., Salt, D.E., 2000. Subcellular localization and speciation of nickel in hyperaccumulator and non-accumulator *Thlaspi* species. Plant Physiology 122, 1343-1354.

Kredich, N.M., Tomkins, G.M., 1966. The enzymic synthesis of L-cysteine in Escherichia coli and Salmonella typhimurium. Journal of Biological Chemistry 241, 4955-4965.

Kretzschmar, R., Voegelin, A., Selim, H.M., Sparks, D.L., 2001. Modeling competitive sorption and release of heavy metals in soils. Heavy metals release in soils. pp. 55-88.

Krishna, M.P., Varghese, R., Arun Babu, V., Jyothy, S., Mohamed Hatha, A.A., 2013. Bioremediation of zinc using *Bacillus* Sp. Isolated from metal-contaminated

industrial zone. Prospects in Bioscience: Addressing the Issues. Springer, India. pp. 11-18.

Krishnamurti, G.S., Pignia, M., Arienzo, M., Violante, A., 2007. Solid phase speciation and phytoavailability of copper in a few representative soils of Italy. Chemical Speciation and Bioavailability 19, 57-67.

Krishnamurti, G.S., Subashchandrabose, S.R., Megharaj, M., Naidu, R., 2015. Assessment of bioavailability of heavy metal pollutants using soil isolates of *Chlorella* Sp. Environmental Science and Pollution Research 22, 8826-8832.

Kucharski, R., Sas Nowosielska, A.S., Ma kowski, E., Japenga, J., Kuperberg, J.M., Pogrzeba, M., Krzyzak, J., 2005. The use of indigenous plant species and calcium phosphate for the stabilization of highly metal polluted sites in southern Poland. Plant Soil 273, 291–305.

Kumar, P.N., Dushenkov, V., Motto, H., Raskin, I., 1995. Phytoextraction: the use of plants to remove heavy metals from soils. Environmental Science and Technology 29, 1232-1238.

Kumar, V., Mahajan, M., Yadav, S.K., 2012. Toxic metals accumulation, tolerance and homeostasis in brassica oilseed species: overview of physiological, biochemical and molecular mechanisms. In: the plant family Brassicaceae; contribution towards phytoremediation. Environmental Pollution 21, 171-211.

Kumar, M.S., 2015. Answering Key Questions Associated with Soil Risk Assessment (August 27, 2015). Available at SSRN: https://ssrn.com/abstract=2652068. [Accessed 22 November 2016].

Küpper, H., Zhao, F.J., McGrath, S.P., 1999. Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. Plant Physiology 119, 305-312.

Kupper, H., Lombi, E., Zhao, F.J., McGrath, S.P., 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212, 75–84.

Küpper, H., Mijovilovich, A., Meyer-Klaucke, W., Kroneck, P.M., 2004. Tissue-and age-dependent differences in the complexation of cadmium and zinc in the cadmium/zinc hyperaccumulator *Thlaspi caerulescens* (Ganges ecotype) revealed by X-ray absorption spectroscopy. Plant Physiology 134, 748-757.

Kupper, H., Anderson, E., 2016. Mechanisms of metal toxicity in plants. Metallomics 8, 269.

Kurepa, J., Paunesku, T., Vogt, S., Arora, H., Rabatic, B.M., Lu, J., Wanzer, M.B., Woloschak, G.E., Smalle, J.A., 2010. Uptake and distribution of ultrasmall anatase TiO_2 alizarin red S nanoconjugates in *Arabidopsis thaliana*. Nano letters 10, 2296-2302.

Langner, P., Mikutta, C., Kretzschmar, R., 2012. Arsenic sequestration by organic sulphur in peat. Nature Geoscience 5, 66-73.

Lanson, B., Marcus, M.A., Fakra, S., Panfili, F., Geoffroy, N., Manceau, A., 2008. Formation of Zn–Ca phyllomanganate nanoparticles in grass roots. Geochimica et Cosmochimica Acta 72, 2478-2490.

Larcher, W., 1995. Physiological plant ecology. Springer, Berlin. pp. 506.

Lasat, M.M., Baker, A.J., Kochian, L.V., 1998. Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. Plant Physiology 118, 875-883.

Latawiec, A.E., Simmons, P., Reid, B.J., 2010. Decision-makers' perspectives on the use of bioaccessibility for risk-based regulation of contaminated land. Environment International 36, 383-389.

Leblanc, M., Petit, D., Deram, A., Robinson, B., Brooks, R.R., 1999. The phytomining and environmental significance of hyperaccumulator of thallium by Iberis intermedia from southern France. Economic Geology 94, 109-113.

Lee, C.W., Choi, J.M., Pak, C.H., 1996. Micronutrient toxicity in seed geranium (Pelargonium×hortorum Baley). Journal of the American Society for Horticultural Science 121, 77–82.

Lee, C.W., Mahendra, S., Zodrow, K., Li, D., Tsai, Y.C., Braam, J., Alvarez, P.J., 2010. Developmental phytotoxicity of metal oxide nanoparticles to *Arabidopsis thaliana*. Environmental Toxicology and Chemistry 29, 669-675.

Lee, M., Yang, M., 2010. Rhizofiltration using sunflower (*Helianthus annuus L*.) and bean (*Phaseolus vulgaris L. var. vulgaris*) to remediate uranium contaminated groundwater. Journal of Hazardous Materials 173, 589-596.

Lee, W.M., An, Y.J., Yoon, H., Kweon, H.K., 2008. Toxicity and bioavailability of copper nanoparticles to the terrestrial plants mug bean (*Phaseolus radiates*) and wheat (*Tritium aestivum*). Plant agar test for water-insoluble nanoparticles. Environmental Toxicology and Chemistry 27, 1915-1921.

Leitenmaier, B., Küpper, H., 2013. Compartmentation and complexation of metals in hyperaccumulator plants. Front Plant Science 4, 13.

Leustek T., Saito, K., 1999. Sulfate transport and assimilation in plants. Plant Physiology 120, 637–643.

Leustek, T., Martin, M.N., Bick, J.A., Davies, J.P., 2000. Pathway and regulation of sulphur metabolism revealed through molecular and genetic studies. Annual Review of Plant Physiology and Plant Molecular Biology 51, 141–165.

Leustek, T., 2002. Sulfate metabolism. The Arabidopsis Book, p. e0017.

Li, K., Ramakrishna, W., 2011. Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth. Journal of Hazardous Materials 189, 531–539.

Li, K.E., Chang, Z.Y., Shen, C.X., Nan, Y., 2015. Toxicity of nanomaterials to plants. In nanotechnology and plant sciences. Springer International Publishing. pp. 101-123.

Li, M., Zhu, L., Lin, D., 2011. Toxicity of ZnO nanoparticles to *Escherichia coli*: mechanism and the influence of medium components Environmental Science Technology 45, 1977–1983.

Li, M., Lin, D., Zhu, L., 2013. Effects of water chemistry on the dissolution of ZnO nanoparticles and their toxicity to *Esherichia coli*. Environmental Pollution 173, 97-102.

Li, P.J., Stagnitti, F., Allinson, G., Turoczy, N., Xiong, X., Peterson, J., 2006. Sorption and fractionation of copper in soil at a sewage irrigation farm in Australia. Communications in Soil Science and Plant Analysis 37, 1031-1042.

Li, X., Gui, X., Rui, Y., Ji, W., Yu, Z., Peng, S., 2014. Bt-transgenic cotton is more sensitive to CeO2 nanoparticles than its parental non-transgenic cotton. Journal of Hazardous Materials 274, 173-180.

Li, W.C., Ye, Z.H., Wong, M.H., 2007. Effects of bacteria on enhanced metal uptake of the Cd/Zn-hyperaccumulating plant*, Sedum alfredii*. Journal of Experimental Botany 58, 4173–4182.

Li, W.C., Ye, Z.H., Wong, M.H., 2010. Metal mobilization and production of short-chain organic acids by rhizosphere bacteria associated with a Cd/Zn hyperaccumulating plant, *Sedum alfredii*. Plant and Soil 326, 453-467.

Liang, H.M., Lin, T.H., Chiou, J.M., Yeh, K.C., 2009. Model evaluation of the phytoextraction potential of heavy metal hyperaccumulators and non-hyperaccumulators. Environmental Pollution 157, 1945–1952.

Lin, D., Xing, B., 2007. Phytotoxicity of nanoparticles: inhibition of seed germination and root growth. Environmental Pollution 150, 243–250.

Lin, D., Xing, B., 2008. Root uptake and phytotoxicity of ZnO nanoparticles. Environmental Science and Technology 42, 5580-5585.

Lin, S., Reppert, J., Hu, Q., Hudson, J.S., Reid, M.L., Ratnikova, T.A. Rao, A.M., Luo, H., Ke, P.C., 2009. Uptake, translocation, and transmission of carbon nanomaterials in rice plants. Small 5, 1128-1132.

Lin, W., Xiao, W., Ning, Z., 2015. Pb, Zn and Cd distribution and migration at historical Zn smelting site. Polish Journal of Environment Studies 24, 575-583.

Lindsay, W.L., 1978. Chemical reactions affecting the availability of micro-nutrients in soils. In mineral nutrition of legumes in tropical and subtropical soils. Proceedings

of a workshop held at CSIRO Cunningham Laboratory, Brisbane, January 1978. CSIRO. pp. 153-167.

Lindsay, W.L., 1979. Chemical equilibria in soils. John Wiley and Sons: New York, USA.

Lindsay, W.L., 1981. Solid phase-solution equilibria in soils. In Chemistry in the soil Environment. American Argon Society. Madison, USA.

Liu, H., Zhang, Z., Hu, L., Gao, N., Sang, L., Liao, M., Ma, R., Xu, F., Fang, X., 2014. New UV-A photodetector based on individual potassium niobate nanowires with high performance. Advance Optical Materials 2, 771-778.

Liu, J.L., Li, Y.L., Zhang, B., Cao, J.L., Cao, Z.G., 2009. Ecological risk of heavy metals in sediments of the Luan river source water. Ecotoxicology 18, 748–758.

Loewen, P.C, Switala, J., Fernando, W.G.D., De Kievit T., 2014. Genome sequence of *Pseudomonas brassicacearum* DF41. Genome Announcements 2, 00390.

Lombi, E., Zhao, F.J., Dunham, S.J., McGrath, S.P., 2001. Phytoremediation of heavy metal contaminated soils: natural hyperaccumulation versus chemically-enhanced phytoextraction. Journal of Environmental Quality 30, 1919–1926.

Lombi, E., Tearall, K.L., Howarth, J.R., Zhao, F.J., Hawkesford, M.J., McGrath, S.P., 2002. Influence of iron status on calcium and zinc uptake by different ecotypes of the hyperaccumulator *Thlaspi caerulescens.* Plant Physiology 128, 1359–1367.

Lombi, E., Hamon, R.E., McGrath, S.P., McLaughlin M.J., 2003. Lability of Cd, Cu, and Zn in polluted soils treated with lime, beringite, and red mud and identification of a non-labile colloidal fraction of metals using isotopic techniques. Environmental Science and Technology 37, 979-984.

Lombi, E., Susini, J., 2009. Synchrotron-based techniques for plant and soil science: opportunities, challenges and future perspectives. Plant Soil 320, 1–35.

Lone, M.I., He, Z., Stoffella, P.J., Yang, X., 2008. Phytoremediation of heavy metal polluted soils and water: progresses and perspectives. Journal of Zhejiang University Science B 9, 210–220.

Loneragan, J.F., Webb, M.J., 1993. Interactions between zinc and other nutrients affecting the growth of plants. In zinc in soils and plants. Springer Netherlands. pp. 119-134.

Long, H.H., Schmidt, D.D., Baldwin, I.T., 2008. Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. PLoS One 3, e2702.

Long, X.X., Yang, X.E., Ni, W.Z., 2002. Current status and perspective on phytoremediation of heavy metal polluted soils. Journal of Applied Ecology 13, 757–762.

Lopez, M.L., Peralta-Videa, J.R., Parsons, J.G., Benitez, T., Gardea-Torresdey, J.L., 2007. Gibberellic acid, kinetin, and the mixture indole–3-acetic acid–kinetin assisted with EDTA-induced lead hyperaccumulation in alfalfa plants. Environmental Science and Technology 41, 8165–8170.

Lopez-Moreno, M.L., De la Rosa G., Hernandez-Viezcas, J.A., Castillo-Michel, H., Botez, C.E., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2010a. Evidence of the differential biotransformation and genotoxicity of ZnO and CeO_2 nanoparticles on soybean (*Glycine max*) plants. Environmental Science and Technology 44, 7315–7320.

López-Moreno, M.L., De la Rosa, G., Hernández-Viezcas, J.A., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2010b. X-ray absorption spectroscopy (XAS) corroboration of the uptake and storage of CeO_2 nanoparticles and assessment of their differential toxicity in four edible plant species. Journal of Agricultural and Food Chemistry 58, 3689-3693.

Lopez-Sanchez, J.F., Sahuquillo, A., Rauret, G., Lachica, M., Barahona, E., Gomez, A., Ure, A.M., Muntau, H., Quevauviller, P.H., 2002. Extraction procedures for soil analysis. Methodologies in Soil and Sediment Fractionation Studies. pp. 28-65.

Lotfy, S.M., Mostafa A.Z., Abdel-Sabour, M.F., 2012. Phytoextraction of cadmium from and zinc contaminated soils. Proceeding of the third international conference on radiation sciences and applications. Journal of Radiation Research and Applied Sciences 5, 490-503.

Lu, L., Tian, S., Zhang, J., Yang, X., Labavitch, J.M., Webb, S.M., Latimer, M., Brown, P.H., 2013. Efficient xylem transport and phloem remobilization of Zn in the hyperaccumulator plant species *Sedum alfredii*. New Phytologist 198, 721-731.

Lubick, N., 2008. Nanosilver toxicity: ions, nanoparticles or both?. Environmental Science and Technology 42, 8617-8617.

Lucas, W.J., Lee, J.Y., 2004. Plasmodesta as a supracellular control network in plants. Nature Reviews Molecular Biology 5, 712-726.

Luo, S., Xu, T., Chen, L., Chen, J., Rao, C., Xiao, X., Wan, Y., Zeng, G., Long, F., Liu, C. and Liu, Y., 2012. Endophyte-assisted promotion of biomass production and metal-uptake of energy crop sweet sorghum by plant-growth-promoting endophyte Bacillus sp. SLS18. Applied Microbiology and Biotechnology 93, 1745-1753.

Lupwayi, N.Z., Rice, W.A., Clayton, G.W., 2000. Endophytic Rhizobia in barley and canola in rotation with field peas. Book of abstracts, 17th North American conference on symbiotic nitrogen fixation. University of Laval, Quebec, Canada. pp 5123-5128.

Lv, J., Zhang, S., Luo, L., Han, W., Zhang, J., Yang, K., Christie, P., 2012. Dissolution and microstructural transformation of ZnO nanoparticles under the influence of phosphate. Environmetal Science and Technology 46, 7215–7221.

Lv, J., Zhang, S., Luo, L., Zhang, J., Yang, K., Christie, P., 2015. Accumulation, speciation and uptake pathway of ZnO nanoparticles in maize. Environmental Science: Nano 2, 68-77.

Lynch, J.M., 1990. Introduction: some consequences of microbial rhizosphere competence for plant and soil. The Rhizosphere. Wiley, Chichester. pp. 1–10.

Lytle, C.M., Lytle, F.W., Yang, N., JinHong, Q., Hansen, D., Zayed, A., Terry, N., 1998. Reduction of Cr (IV) to Cr (III) by wetland plants. Potential for in situ heavy metal detoxification. Environmental Science and Technology 32, 3087-3093.

Ma, C., White, J.C., Dhankher, O.P., Xing, B., 2015. Metal-based nanotoxicity and detoxification pathways in higher plants. Environmental Science and Technology 49, 7109-7122.

Ma, H.B., Williams, P.L., Diamond, S.A., 2013. Ecotoxicity of manufactured ZnO nanoparticles—a review. Environmental Pollution 172, 76–85.

Ma, L.Q., Rao, G.N., 1997. Chemical fractionation of cadmium, copper, nickel and zinc in contaminated soils. Journal of Environmental Quality 26, 259–264.

Ma, L.Q., Komar, K.M., Tu, C., Wu, Q., 2001. A fern that hyperaccumulates arsenic. Nature 409, 579.

Ma, R., Levard, C., Michel, F.M., Brown, G.E., Lowry, G.V., 2013. Sulfidation mechanism for zinc oxide nanoparticles and the effect of sulfidation on their solubility. Environmental Science and Technology 47, 2527–2534.

Ma, X., Geiser-Lee, J., Deng, Y., Kolmakov, A., 2010. Interactions between engineered nanoparticles (ENPs) and plants: phytotoxicity, uptake and accumulation. Science of the Total Environment 408, 3053–3061.

Ma, X., Gurung, A., Deng, Y., 2013. Phytotoxicity and uptake of nanoscale zero-valent iron (nZVI) by two plant species. Science of the Total Environment 443, 844–849.

Ma, Y., Kuang, L., He, X., Bai, W., Ding, Y., Zhang, Z., Zhao, Y., Chai, Z., 2010. Effects of rare earth oxide nanoparticles on root elongation of plants. Chemosphere 78, 273-279.

Ma, Y., Prasad, M.N.V., Rajkumar, M., Freitas H., 2011. Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnology Advances 29, 248–258.

Ma, Y., Lombi, E., McLaughlin, M.J., Oliver, I.W., Nolan, A.L., Oorts, K., Smolders, E., 2013. Aging of nickel added to soils as predicted by soil pH and time. Chemosphere 92, 962-968.

Ma, Y., Mani Rajkumar, M., Rocha, I., Oliveira, R., Freitas, H., 2015a. Serpentine bacteria influence metal translocation and bioconcentration of *Brassica juncea* and

Ricinus communis grown in multi-metal polluted soils. Frontiers in Plant Science 5, 757.

Ma, Y., Oliveira, R.S., Wu, L., Luo, Y., Rajkumar, M., Rochas, I., Frietas, H., 2015b. Inoculation with metal mobilizing plant growth promoting rhizobacterium Bacillus sp.SC2b and its role in rhizoremediation. Journal of Toxicology and Environmental Health, Part A 78, 931-944.

Ma, Y.B., Uren, N.C., 2006. Effect of aging on the availability of zinc added to a calcareous clay soil. Nutrient Cycling in Agroecosystem 76, 11-18.

MacCormack, T., Goss, G., 2008. Identifying and predicting biological risks associated with manufactured nanoparticles in aquatic ecosystems. Journal of Industrial Ecology 12, 286 – 296.

Mahajan, P., Dhoke, S.K., Khanna, A.S., 2011. Effect of nano-ZnO particle suspension on growth of mung (Vigna radiata) and gram (Cicer arietinum) seedlings using plant agar method. Journal of Nanotechnology 2011.

Mahapatra, N.R., Banerjee, P.C., 1996. Extreme tolerance to cadmium and high resistance to copper, nickel and zinc in different *Acidiphilium* strains. Letters in Applied Microbiology 23, 393-397.

Mahar, A., Wang, P., Ali, A., Awasthi, M.K., Lahori, A.H., Wang, Q., Li, R., Zhang, Z., 2016. Challenges and opportunities in the phytoremediation of heavy metals contaminated soils: A review. Ecotoxicology and Environmental Safety 126, 111-121.

Mahdavi, S., Jalali, M., Afkhami, A., 2012. Removal of heavy metals from aqueous solutions using Fe_3O_4, ZnO, and CuO nanoparticles. Journal of Nanoparticles Research 14, 846.

Maitra, S., Banerjee, P.K., 2015. Estimation of plant growth promoting potential of a nickel accumulating isolate obtained from dhapa industrial wasteland (Kolkata, India) soil on Indian yellow mustard (*Brassica hirta*). International Journal of Current Microbiology and Applied Science 4, 765-772.

Malandrino, M., Abollino, O, Buoso, S., Giacomino, A., La Gioia, C., Mentasti, E., 2011. Accumulation of heavy metals from contaminated soil to plants and evaluation of soil remediation by vermiculite. Chemosphere 82, 169–178.

Manara, A., 2012. Plant responses to heavy metal toxicity. Plants and heavy metals. Springer, Netherland. pp. 27-53.

Mandal, S.M., Dey, S., Ray, B., Pati, B.R., 2007a. Production and composition of extracellular polysaccharides synthesised by a rhizobium isolate of *Vigna mungo (L.) Heppe*. Biotechnology Letter 29, 1271-1275.

Mandal, S.M., Mondal, K.C., Dey, S., Pati, B.R., 2007b. Optimization of cultural and nutritional conditions for indol - 3- acetic acid production by a Rhizobium sp. Isolated from root nodules of *Vigna mungo (L) hepper.* Research Journal of Microbiology 2, 239-246.

Mani, D., Kumar, C., Patel, N.K., 2015. Integrated micro-biochemical approach for phytoremediation of cadmium and zinc contaminated soils. Ecotoxicology and Environmental Safety 111, 86–95.

Manke, A., Wang, L., Rojanasakul, Y., 2013. Mechanisms of nanoparticle-induced oxidative stress and toxicity. Biomed Research International 2013, 15.

Marchiol, L., Assolari, S., Sacco, P., Zerbi, G., 2004. Phytoextraction of heavy metals by canola (*Brassica napus*) and radish (*Raphanus sativus*) grown on multi contaminated soil. Environmental Pollution 132, 21-27.

Marmiroli, M., Pagano, L., Savo Sardaro, M.L., Villani, M., Marmiroli, N., 2014. Genome-wide approach in *Arabidopsis thaliana* to assess the toxicity of cadmium sulfide quantum dots. Environmental Science and Technology 48, 5902-5909.

Marques, A.P.G.C., Rangel, A.O.S.S., Castro, P.M.L., 2009. Remediation of heavy metal contaminated soils: Phytoremediation as a potentially promising clean-up technology. Environmental Science and Technology 39, 622–654.

Marschner, H., 1995. Mineral Nutrition of Higher Plants. Academic Press, London.

Marschner, H., 1986. Mineral nutrition of higher plants. Academic Press London, UK.

Marschner, H., Römheld, V., Horst, W.J., Martin, P., 1986. Root-induced changes in the rhizosphere: Importance for the mineral nutrition of plants. Pflanzenernaehr Bodenk 149, 441–456.

Martin, J.M., Nirel, P., Thomas, A.J., 1987. Sequential extraction techniques: Promises and problems. Marine Chemistry 22, 313-341.

Martinez-Fernandez, D., Vitkova, M., Bernal, M.P., Komarek, M., 2015. Effects of nano-maghemite on trace element accumulation and drought response of *Helianthus annuus L.* in a contaminated mine soil. Water Air Soil Pollution 226, 1–4.

Martinez-Romero, E., Gutierrez-Zamora, M.L., Estrada, P., Caballero-Mellado, J., Hernandez-Lucas, I., 2000. Natural endophytic association between *Rhizobium Etli* and maize. Book of abstracts, 17th North American conference on symbiotic nitrogen fixation, July 2000. University of Laval, Quebec, Canada. pp. 51.

Maurer-Jones, M.A., Gunsolus, I.L., Murphy, C.J., Haynes, C.L., 2013. Toxicity of Engineered Nanoparticles in the Environment. Analytical Chemistry 85, 3036–3049.

Mayak, S., Tirosh, T., Glick, B.R., 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiology Biochemistry 42, 565-572.

McCaffery, D., Bambach, R., Haskins, B., 2009. *Brassica juncea* in north-western NSW. Primefacts, DPI, NSW. Available online at: http// www.nvtonline.com.au. [Accessed 9 April 2015].

McCall, K.A., Huang, C., Fierke, C.A., 2000. Function and mechanism of zinc metalloenzymes. Journal of Nutrition 130, 1437-1446.

McGrath, S.P., Shen, Z.G., Zhao, F.J., 1997. Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. Plant Soil 188, 153–159.

McGrath, S.P., Sanders, J.R., Shalaby, M.H., 1988. The effects of soil organic matter levels on soil solution concentrations and extractabilities of manganese zinc and copper. Geoderma 42, 177-188.

McGrath, S.P., Zhao, F.J., 2003. Phytoextraction of metals and metalloids from contaminated soils. Current Opinion in Biotechnology 14, 277-282.

McLaren, R.G., Crawford, D.V., 1973. Studies on soil copper: The fractionation of copper in soils. Journal of Soil Science 24, 172–181.

McLean, J.E., Bledsoe, B.E., 1992. Ground water issue. Behaviour of metals in soils. United States Environmental Protection Agency Office of Solid Waste and Emergency Response. EPA/540/S-92/018.

Mclnroym, J.A., Kloepper, J.W., 1995. Survey of indigenous endophytes from cotton and sweet corn. Plant Soil 173, 337 – 342.

McNear Jr, D.H., 2013. The Rhizosphere - roots, soil and everything in between. Nature Education Knowledge 4, 1.

Meda, A.R., Scheuermann, E.B., Frechsl, U.E., Rough, P.D., 2007. Iron acquisition by phytosiderophores contributes to cadmium tolerance. Plant Physiology 143, 1761–1773.

Medas, D., De Giudici, G., Casu, M.A., Musu, E., Gianoncelli, A., Iadecola, A., Meneghini, C., Tamburini, E., Sprocati, A.R., Turnau, K., Lattanzi, P., 2015. Microscopic processes ruling the bioavailability of Zn to roots of Euphorbia pithyusa L. Pioneer plant. Environmental Science and Technology 49, 1400-1408.

Meharg, A.A., Cairney, J.W., 2000. Ectomycorrhizas—extending the capabilities of rhizosphere remediation?. Soil Biology and Biochemistry 32, 1475-1484.

Meharg, A.A., Hartley-Whitaker, J., 2002. Arsenic uptake and metabolism in arsenic resistant and non- resistant plant species. New Phytologist 154, 29-42.

Mei, B.J., Puryear, J.D., Newton, R.J., 2002. Assessment of Cr tolerance and accumulation in selected plant species. Plant and Soil 247, 223-231.

Meyers, D.E.R., Auchterlonie, G.J., Webb R.I., Wood, B., 2008. Uptake and localisation of lead in the root system of *Brassica juncea*. Environmental Pollution 153, 323–332.

Mhatre, G.N., Pankhurst, C.E., 1997. Bioindicators to detect contamination of soils with special reference to heavy metals. Biological indicators of soil health, CAB International, Wallingford, England. pp. 349-369.

Miao, A., Schwehr, K.A., Xu, C., Zhang, S., 2009. The algal toxicity of silver engineered nanoparticles and detoxification by exopolymeric substances. Environmental Pollution 157, 3034–3041.

Miller, R.J., Lenihan, H.S., Muller, E.B., Tseng, N., Hanna, S.K., Keller, A.A., 2010. Impacts of metal oxide nanoparticles on marine phytoplankton. Environmental Science and Technology 44, 7329–7334.

Mills, R.F., Krijger, G.C., Baccarini, P.J., Hall, J.L., Williams, L.E., 2003. Functional expression of AtHMA4, a P1B-type ATPase of the Zn/Co/Pb subclass. The Plant Journal 35, 164–176.

Milner, M.J., Kochian, L.V., 2008. Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens as* a model system. Annals of Botany 102, 3-13.

Ministry of Agriculture, Fisheries and Food., 1993. Review of the rules for sewage sludge application to agricultural land: Soil fertility aspects of potentially toxic elements. Independent scientific committee on soil fertility sspects of sewage sludge use in agriculture. PB 1561. MAFF Publisher, London.

Minz, D., Ofek, M, Hadar, Y., 2013. Plant rhizosphere microbial communities. The Prokaryotes: Prokaryotic communities and ecophysiology. Springer-Verlag, Berlin. pp. 57–84.

Miralles, P., Johnson, E., Church, T.L., Harris, A.T., 2012. Multiwalled carbon nanotubes in alfalfa and wheat: toxicology and uptake. Journal of the Royal Society Interface 9, 3514-3527.

Mirshekali, H., Hashem, H., Amirnia, R., Verdiloo, H.K., 2012. Effects of zinc toxicity on plant productivity, chlorophyll and Zn contents of sorghum (*Sorghun bicolor*) and common Iambsquarter (*Chenopodium album*). International Journal of Agriculture Research and Review 2, 247-254.

Misko, A.L., Germida, J.J., 2002. Taxonomy and functional diversity of Pseudomonads isolated from the root of field grown canola. FEMS Microbial Ecology 42, 399-407.

Mitra, G.N., 2015. Regulation of nutrient uptake by plants: A biochemical and molecular approach. Springer. pp. 130.

Mitrano, D.M., Motellier, S., Clavaguera, S., Nowack, B., 2015. Review of nanomaterial aging and transformations through the life cycle of nano-enhanced products. Environmental International 77, 132-147.

Mohammadi, R., Maali-Amiri, R., Mantri, N.L., 2014. Effect of TiO2 nanoparticles on oxidative damage and antioxidant defense systems in chickpea seedlings during cold stress. Russian Journal of Plant Physiology 61, 768-775.

Monica, R.C., Cremonini, R., 2009. Nanoparticles and higher plants. Caryologia 62, 161–165.

Monsant, A.C., Kappen, P., Wang, Y., Pigram, P.J., Baker, A.J., Tang, C., 2011. In vivo speciation of zinc in *Noccaea caerulescens* in response to nitrogen form and zinc exposure. Plant and Soil 348, 167-183.

Moore, G.L., 1989. Introduction to inductively coupled plasma atomic emission spectrometry. Amsterdam.

Moore, M.N., 2006. Do nanoparticles present ecotoxicological risks for the health of the aquatic environment. Environment International 32, 967–976.

Morales, M.I., Rico, C.M., Hernandez-Viezcas, J.A., Nunez, J.E., Barrios, A.C., Tafoya, A., Flores-Marges, J.P., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2013. Toxicity assessment of cerium oxide nanoparticles in cilantro (*Coriandrum sativum L*.) plants grown in organic soil. Journal of Agricultural and Food Chemistry 61, 6224-6230.

Moreau, J.W., Weber, P.K., Martin, M.C., Gilbert, B., Hutcheon, I.D., Banfield, J.F., 2007. Extracellular proteins limit the dispersal of biogenic nanoparticles. Science 316, 1600-1603.

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt N., Vavasseur, A., Richaud, P., 2009. AtHMA3, a P1B-type ATPase allowing Cd/Zn/Co/Pb vacuolar storage in Arabidopsis. Plant Physiology 149, 894–904.

Morones, J.R., Elechiguerra, J.L., Camacho, A., Holt, K., Kouri, J.B., Ramırez, J.T., Yacaman, M.J., 2007. The bactericidal effect of silver nanoparticles. Nanotechnology 16, 2346–2353.

Mosselmans, F., Quinn, P., Dent, A., Cavill, S., Diaz-moreno, S., Peach, A., Leicester, P., Keylock, S., Gregory, S., Atkinson, K., Rose, J.R., 2009. I18--the microfocus spectroscopy beamline at the Diamond Light Source. Journal of Synchrotron Radiation 16, 818-824.

Mossop, K.F., Davidson C.M., 2003. Comparison of original and modified BCR sequential extraction procedures for the fractionation of copper, iron, lead, manganese and zinc in soil and sediments. Analytica Chimica Acta 478, 111-118.

Mudunkotuwa, I.A., Rupasinghe, T., Wu, C.M., Grassian, V.H., 2011. Dissolution of ZnO nanoparticles at circumneutral pH: a study of size effects in the presence and absence of citric acid. Langmuir 28, 396-403.

Mueller, N.C., Nowack, M., 2008. Exposure modelling of engineered nanoparticles in the environment. Environmental Science and Technology 42, 4447-4453.

Mueller, N.C., Nowack, B., 2010. Nanoparticles for remediation: Solving big problems with little particles. Elements 6, 395–400.

Mukherjee, A., Pokhrel, S., Bandyopadhyay, S., Mädler, L., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2014. A soil mediated phyto-toxicological study of iron doped zinc oxide nanoparticles (Fe@ ZnO) in green peas (Pisum sativum L.). Chemical Engineering Journal 258, 394-401.

Myers, J.A., Curtis, B.S., Curtis, W.R., 2013. Improving accuracy of cell and chromophore concentration measurements using optical density. BMC Biophysics 6, 4.

Nadler, K.D., Herron, H., Granick, S., 1972. Development of chlorophyll and Hill activity. Plant Physiology 49, 388 – 393.

Nagajyoti, P.C., Lee, K.D., Sreekanth, T.V.M., 2010. Heavy metals, occurrence and toxicity for plants: a review. Environmental Chemistry Letters 8, 199-216.

Nair, R., Varghese, S.H., Nair, B.G., Maekawa, T., Yoshida, Y., Kumar, D.S., 2010. Nanoparticulate material delivery to plants. Plant Science 179, 154-163.

Nair, P.M.G., Kim, S.H., Chung, I.M., 2014. Copper oxide nanoparticle toxicity in mung bean (Vigna radiata L.) seedlings: physiological and molecular level responses of in vitro grown plants. Acta Physiologiae Plantarum 36, 2947-2958.

Nair, P.M.G., Chung, I.M., 2015. The responses of germinating seedlings of green peas to copper oxide nanoparticles. Biologia Plantarum 59, 591-595.

Nanotechnology. ASTM E2456-06. Available online at: http://www.astm.org/Standards/E2456.htm. [Accessed 12 January 2016].

Nasch, T., Jeitschko, W., 1999. Niobium and molybdenum compounds with high zinc content: $NbZn_3$, $NbZn_{16}$, and $MoZn_{20.44}$. Journal of Solid State Chemistry 143, 95-103.

Natural and Induced Phytoextraction. [Image online] Available at: <http://www.bnl.gov/erd/Peconic/Factsheet/Phytoextract.pdf> [Accessed 4 April 2012].

Navarro, E., Baun, A., Behra, R., Hartmann, N.B., Filser, J., Miao, A.J., Quigg, A., Santschi, P.H., Sigg, L., 2008. Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. Ecotoxicology 17, 372-386.

Nearing, M.M., Koch, I., Reimer, K.J., 2014. Complementary arsenic speciation methods: A review. Spectrochimica Acta Part B: Atomic Spectroscopy 99, 150-162.

Negri, M.C., Gatliff, E.G., Quinn, J.J., Hinchman, R.R., 2003. Root development and rooting at depths. Phytoremediation: transformation and control of contaminants. John Wiley and Sons. pp. 233-262.

Nel, A., Xia, T., Moedler, L., Li, N., 2006. Toxic material at nano level. Science 311, 622-627.

Nemati, K., Abu Bakar, N.K., Sobhanzadeh, E., Abas, M.R., 2009. A modification of the BCR sequential extraction procedure to investigate the potential mobility of copper and zinc in shrimp aquaculture sludge. Microchemical Journal 92, 165-169.

Nemati, K., Abu Bakar, N.K., Abas, M.R., Sobhanzadeh, E., 2011. Speciation of heavy metals by modified BCR sequential extraction procedure in different depths of sediments from Sungai Buloh, Selangor, Malaysia. Journal of Hazardous Materials 192, 402– 410.

Nesbitt, H.W., 1992. Diagenesis and metasomatism of weathering profile, with emphasis on Precambrian paleosols. Weathering, Soils and Paleosols 2, 127.

Newman, L.A., Strand, S.E., Choe, N., Duffy, J., Ekuan, G., Ruszaj, M., Shurtleff, B.B., Wilmoth, J., Heilman, P., Gordon, M.P., 1997. Uptake and biotransformation of trichloroethylene by hybrid poplars. Environmental Science and Technology 31, 1062–1067.

NFESC., 2000. Guide for incorporating bioavailability adjustments into human health and ecological risk assessments at US navy and Marine Corps facilities, Part 1: overview of metals bioavailability, User's Guide UG-2041-ENV.

Ng, S.K., Bloomfield, C., 1962. The effect of flooding and aeration on the mobility of certain trace elements in soils. Plant and Soil 16, 108–135.

Nicholson, F.A., Smith, S.R., Alloway, B.J., Carlton-Smith, C., Chambers, B.J., 2003. An inventory of heavy metals inputs to agricultural soils in England and Wales. Science of the Total Environment 311, 205-219.

Nicks, L., Chambers, M.F., 1994. Nickel farming. Discovery magazine. pp. 19.

Nie, Z., Petukhova, A., Kumacheva, E., 2010. Properties and emerging applications of self-assembled structures made from inorganic nanoparticles. Nature Nanotechnology 5, 15-25.

Noel, T.C., Sheng, C., Yost, C.K., Pharis, R.P., Hynes, M.F., 1996. *Rhizobium leguminosarum* as a plant growth –promoting rhizobacterium: direct promotion of canola and lettuce. Canadian Journal of Microbiology 42, 279-283.

Norvell, W.A., Welch, R.M., 1993. Growth and nutrient uptake by barley: Studies using an N (2-Hydroxyethyle) ethylenedinitrilotriacetic acid buffered nutrient solution technique. Zinc ion requirements. Plant Physiology 101, 619-625.

Novo, L.A.B., Gonzalez, L., 2013. The effects of variable soil moisture on the phytoextraction of Cd and Zn by *Brassica juncea*. Fresenius Environment Bulletin 22, 299–304.

Novo, L.A.B., Covelo, E.F., González, L., 2014. Effect of salinity on zinc uptake by *Brassica juncea*. International Journal of Phytoremediation 16, 704-718.

Nowack, B., Bucheli, T.D., 2007. Occurrence, behaviour and effects of nanoparticles in the environment. Environmental Pollution 150, 5-22.

Nowack, B., 2008. Pollution prevention and treatment using nanotechnology. Wiley-VCS Verlag GmbH and Company, Weinheim. pp. 1-15.

Nowack, B., Ranville, J., Diamond, S., Gallego-Urrea, J., Metcalfe, C., Rose, J., Horne, N., Koelmans, A. A., Klaine, S.J., 2012. Potential scenarios for nanomaterial release and subsequent alteration in the environment. Environmental Toxicology Chemistry 31, 50-59.

Nye, P.H., 1981. Changes of pH across the rhizosphere induced by roots. Plant Soil 61, 7–26.

Oberdörster, G., 2010. Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. Journal of Internal Medicine 267, 89-105.

Oberdorster, G., Stone, V., Donaldson, K., 2007. Toxicology of nanoparticles: A historical perspective. Nanotoxicology 1, 2-25.

Okoro, H.K., Fatoki, O.S., Adekola, F.A., Ximba, B.J., Snyman, R.G., 2012. A review of sequential extraction procedures for heavy metals speciation in soil and sediments. Open Scientific Reports 1, 181.

Ordookhani, K., 2011. Investigation of PGPR on antioxidant activity of essential oil and microelement contents of sweet basil. Advances in Environmental Biology 5, 1114-1119.

Ortet, P., Barakat, M., Lalaouna, D., Fochesato, S., Barbe, V., Vacherie, B., Santaella, C., Heulin, T., Achouak, W., 2011. Complete genome sequence of a beneficial plant root-associated bacterium, *Pseudomonas brassicacearum*. Journal of Bacteriology 193, 3146–3147.

Osman, I.F., Baumgartner, A., Cemeli, E., Fletcher, J.N., Anderson D., 2010. Genotoxicity and cytotoxicity of zinc oxide and titanium dioxide in HEp-2 cells. Nanomedicine 5, 1193–1203.

Oves, M., Khan, M.S., Zaidi, A., Ahmad, E., 2012. Soil contamination, nutritive value and human health risk assessment of heavy metals: an overview. Toxicity of heavy

metals to legumes and bioremediation. Zaidi, A., Wani, P.A., Khan, M.S. Springer-Verlag. pp. 1–27.

Pachapur, V.L., Larios, A.D., Cledón, M., Brar, S.K., Verma, M., Surampalli, R.Y., 2016. Behavior and characterization of titanium dioxide and silver nanoparticles in soils. Science of the Total Environment 563, 933-943.

Pajuelo, E., Rodrıguez-Llorente, I.D., Lafuente, A., Caviedes, M.A., 2011. Legume–Rhizobium symbioses as a tool for bioremediation of heavy metal polluted soils. Biomanagement of Metal-Contaminated Soils 20, 95–123.

Pan, B., Xing, B., 2012. Application and implications of manufactured nanoparticles in soils: a review. European Journal of Soil Science 63, 437-456.

Pan, H., Zhang, Y., He, G.X., Katagori, N., Chen, H., 2014. A comparison of conventional methods for the quantification of bacterial cells after exposure to metal oxide nanoparticles. BMC Microbiology 14, 1.

Panfili, F., Manceau, A., Sarret, G., Spadini, L., Kirpichtchikova, T., Bert, V., Laboudigue, A., Marcus, M.A., Ahamdach, N., Libert, M.F., 2005. The effect of phytostabilization on Zn speciation in a dredged contaminated sediment using scanning electron microscopy, X-ray fluorescence, EXAFS spectroscopy, and principal components analysis. Geochimica et Cosmochimica Acta 69, 2265-2284.

Papp, T., Schiffmann, D., Weiss, D., Castranova, V., Vallyathan, V., Rahman, Q., 2008. Human health implications of nanomaterial exposure. Nanotoxicology 2, 9-27.

Park, J.H., Lamb, D., Paneerselvam, P., Choppala, G., Bolan, N., Chung, J.W., 2011. Role of organic amendments on enhanced bioremediation of heavy metal (loid) contaminated soils. Journal of Hazardous Materials 185, 549-574.

Parker, M.B., Gaines, T.P., Walker, M.E., Plank, C.O., Davis-Carter, J.G., 1990. Soil zinc and pH effects on leaf zinc and the interaction of leaf calcium and zinc on zinc toxicity of peanuts. Communications in Soil Science and Plant Analysis 21, 2319-2332.

Parsaeimehr, A., Alizadeh, O., Jafari, B., 2009. Applying *Azespiriliom* bacteria and interaction of it with *stereptomyces* spp due biological control on wheat (*Triticum asstivum*) sustainable culture. American-Eurasian Journal of Sustainable Agriculture 3, 622-625.

Parsons, J.G., Aldrich, M.V., Gardea-Torresdey, J.L., 2002. Environmental and biological applications of extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge structure (XANES) spectroscopies. Applied Spectroscopy Reviews 37, 187-222.

Patil, S., Shedbalkar, U.U., Truskewycz, A., Chopaded, B.A., Ball, A.S., 2016. Nanoparticles for environmental clean-up: A review of potential risks and emerging solutions. Environmental Technology and Innovation 5, 10-21.

Peck, A.W., McDonald, G.K., 2010. Adequate zinc nutrition alleviates the adverse effects of heat stress in bread wheat. Plant Soil 337, 355-374.

Peijnenburg, W.J.G., Jager, T., 2003. Monitoring approaches to assess bioaccessibility and bioavailability of metals: Matrix issues. Ecotoxicology and Environmental Safety 56, 63–77.

Peijnenburg, W.J., Zablotskaja, M., Vijver, M.G., 2007. Monitoring metals in terrestrial environments within a bioavailability framework and a focus on soil extraction. Ecotoxicology and Environmental Safety 67, 163- 179.

Peltier, E., Dahl, A.L., Gaillard, J.F., 2005. Metal speciation in anoxic sediments: When sulfides can be construed as oxides. Environmental Science and Technology 39, 311-316.

Peralta-Videa, J.R., Zhao, L., Lopez-Moreno, M.L., de la Rosa, G., Hong, J., Gardea-Torresdey, J.L., 2011. Nanomaterials and the environment: a review for the biennium 2008–2010. Journal of Hazardous Materials 186, 1-15.

Percival, H.J., 2003. Soil and soil solution chemistry of a New Zealand pasture soil amended with heavy metal-containing sewage sludge. Australian Journal of Soil Research 41, 1-17.

Perkin-Elmer., 1997. Perkin Elmer lnc Software.

Petosa, A.R., Jaisi, D.P., Quevedo, I.V., Elimelech, M., Tufenkji, N., 2010. Aggregation and deposition of engineered nanomaterials in aquatic environments: Role of physicochemical interaction. Environmental Science and Technology 44, 6532-6549.

Petruzzelli, G., Pedron, F., Barbafieri, M., Tassi, E., gorini, F., Rosellini, I., 2011. Enhanced Bioavailable contaminant stripping: a case study of Hg contaminated soil. Chemical Engineering Transaction 28, 211-216.

Phieler, R., Merten, D., Roth, M., Buchel, G., Kothe, E., 2015. Phytoremediation using microbially mediated metal accumulation in *Sorghum bicolor*. Environmental Science and Pollution Research 22, 19408-19416.

Pickering, I.J., Prince, R.C., George, M.J., Smith, R.D., George, G.N., Salt, D.E., 2000. Reduction and coordination of arsenic in Indian mustard. Plant Physiology 122, 1171-1177.

Pickering, W.F., 1980. Zinc interaction with soil and sediment components. In Zinc in Environemnt. Ecological cycling. John Wiley and Sons. New York, USA. pp. 71-112.

Pieterse, C.M., Zamioudis, C., Berendsen, R.L., Weller, D.M., Van Wees, S.C., Bakker, P.A., 2014. Induced systemic resistance by beneficial microbes. Annual Review of Phytopathology 52, 347-375.

Pignatello, J.J, Xing, B., 1996. Mechanisms of slow sorption of organic chemicals to natural particles. Environmental Science and Technology 30, 1-10.

Pilon-Smits, E., 2005. Phytoremediation. Annual Review of Plant Biology 56, 15–39.
Plathe, K.L., Von Der Kammer, F., Hassellöv, M., Moore, J.N., Murayama, M., Hofmann, T., Hochella, M.F., 2013. The role of nanominerals and mineral nanoparticles in the transport of toxic trace metals: Field-flow fractionation and analytical TEM analyses after nanoparticle isolation and density separation. Geochimica et Cosmochimica Acta 102, 213-225.

Prapagdee, B., Chanprasert, M., Mongkolsuk, S., 2013. Bioaugmentation with cadmium-resistant plant growth-promoting rhizobacteria to assist cadmium phytoextraction by *Helianthus annuus*. Chemosphere 92, 659–666.

Prasad, K.V.S.K., Pardha Saradhi, P., 1995. Effect of zinc on free radical and proline in *Brassica juncea* and *Cajanus cajan*. Phytochemistry 39, 45–47.

Prasad, K.V.S.K., Saradhi, P.P., Sharmila, P., 1999. Concerted action of antioxidant enzymes and curtailed growth under zinc toxicity in *Brassica juncea*. Environmental and Experimental Botany 42, 1-10.

Prasad, M.N.V., Freitas, H.M., De, O., 2003. Metal hyperaccumulation in plants – biodiversity prospecting for phytoremediation technology. Electronic Journal of Biotechnology 6, 285–321.

Prasad, T.N.V., Sudhakar, P., Sreenivasulu, Y., Latha, P., Munaswamy, V., Reddy, K.R., Sreeprasad, T.S., Sajanlal, P.R., Pradeep, T., 2012. Effect of nanoscale zinc oxide particles on the germination, growth and yield of peanut. Journal of Plant Nutrition 35, 905-927.

Priester, J.H., Ge, Y., Mielke, R.E., Horst, A.M., Moritz, S.C., Espinosa, K., Gelb, J., Walker, S.L., Nisbet, R.M., An, Y.J., Schimel, J.P., 2012. Soybean susceptibility to manufactured nanomaterials with evidence for food quality and soil fertility interruption. Proceedings of the National Academy of Sciences 109, E2451-E2456.

Prokop, Z., Cupr, P., Zlevorova-Zlamalikova, V., Komarek, J., Dusek, L., Holoubek, I., 2003. Mobility, bioavailability, and toxic effects of cadmium in soil samples. Environmental Research 91, 119-126.

Pueyo, M., Rauret, G., Luck, D., Yli-Halla, M., Muntau, H., Quevauviller, P., Lopez-Sanchez, J.F., 2001. Certification of the extractable contents of Cd, Cr, Cu, Ni, Pb and Zn in a fresh water sediment following a collaboratively tested and optimised three-step sequential extraction procedure. Journal of Environmental Monitoring 3, 243-250.

Pulford, I. D., 2007. The Chemistry of the solid earth. In principles of environmental chemistry. The Royal Society of Chemistry.

Punshon, T., Jackson, B.P., Lanzirotti, A., Hopkins, W.A., Bertsch, P.M., Burger, J., 2005. Application of synchrotron x-ray microbeam spectroscopy to the determination of metal distribution and speciation in biological tissues. Spectroscopy Letters 38, 343-363.

Purakayastha, T.J., Viswanath, T., Bhadraray, S., Chhonkar, P. K., Adhikari, P.A., Suribabu, K., 2008. Phytoextraction of zinc, copper, nickel and lead from a contaminated soil by different species of *Brassica*. International Journal of Phytoremediation 1, 61–72.

Puschenreiter, M., Wieczorek, S., Horak, O., Wenzel, W.W., 2003 Chemical changes in the rhizosphere of metal hyperaccumulator and excluder *Thlaspi* species. Journal of Plant Nutrition and Soil Science 166, 579–584.

Puschenreiter, M., Schnepf, A., Millan, I.M., Fitz, W.J., Horak, O., Klepp, J., Schrefl, T., Lombi, E., Wenzel, W.W., 2005. Changes of Ni biogeochemistry in the rhizosphere of the hyperaccumulator *Thlaspi goesingense*. Plant and Soil 271, 205-218.

Putnam, D.H., Oplinger, E.S., Hicks, D.R., Durgan, B.R., Noetzel, D.M., Meronuck, R.A., Doll, J.D., Schulte, E.E., 1990. Sunflower. Alternative Field Crops Manual. University of Wisconsin, Cooperative Extension, Madison. Available at: http://www.hort.purdue.edu/newcrop/afcm/sunflower.html. [Accessed 16 December 2015]

Qiao, L., Ho, G., 1996. The effect of clay amendment on speciation of heavy metals in sewage sludge. Water Science and Technology 34, 413-420.

Qiao, X., He, Y., Wang, Z., Li, X., Zhang, K., Zeng, H., 2014. Effect of foliar spray of zinc on chloroplast b-carbonic anhydrase expression and enzyme activity in rice (*Oryza sativa* L.) leaves. Acta Physiologia Plantarum 36, 263-272.

Qu, J., Luo, C., Cong, Q., Yuan, X., 2012. A new insight into recycling of hyperaccumualtor: synthesis of the mixed Cu and Zn oxide nanoparticles using *Brassica juncea*. International Journal of Phytoremediation 14, 854-860.

Quigg, A., Chin, W.C., Chen, C.S., Zhang, S., Jiang, Y., Miao, A.J., Schwehr, K.A., Xu, C., Santschi, P.H., 2013. Direct and indirect toxic effects of engineered nanoparticles on algae: role of natural organic matter. ACS Sustainable Chemistry and Engineering 1, 686-702.

Raboy, V., Young, K.A., Dorsch, J.A. and Cook, A., 2001. Genetics and breeding of seed phosphorus and phytic acid. Journal of Plant Physiology 158, 489-497.

Rahdar, A., Arbabi, V., Ghanbari, H., 2012. Study of electro-optical properties of ZnS nanoparticles prepared by colloidal particles method. International Journal of Chemical and Biological Engineering 6, 81-83.

Rajeshwari, A., Kavitha, S., Alex, S.A., Kumar, D., Mukherjee, A., Chandrasekaran, N., Mukherjee, A., 2015. Cytotoxicity of aluminum oxide nanoparticles on Allium cepa root tip—effects of oxidative stress generation and biouptake. Environmental Science and Pollution Research 22, 11057-11066.

Rajkumar, M., Freitas, M., 2008. Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. Chemosphere 71, 834–842.

Rajkumar, M., Ae, N., Prasad, M.N.V., Frietas, H., 2010. Potential of siderophores producing bacteria for improving heavy metal phytoextraction. Trends in Biotechnology 28, 142-149.

Rajkumar, M., Sandhya, S., Prasad, M.N.V., Freitas, H., 2012. Perspectives of plant – associated microbes in heavy metal phytoremediation. Biotechnology Advances 30, 1562-1574.

Rajkumar, M., Ma, Y., Frietas, H., 2013. Improvement of Ni phytostabilization by inoculation of Ni resistant Bacillus megaterium SR28C. Journal of Environmental Management 128, 973-980.

Rao, S., Shekhawat, G.S., 2014. Toxicity of ZnO engineered nanoparticles and evaluation of their effect on growth, metabolism and tissue specific accumulation in *Brassica juncea.* Journal of Environmental Chemical Engineering 2, 105-114.

Rascio, N., Navari-Izzo, F., 2011. Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? Plant Science 180, 169-181.

Raskar, S.V., Laware, S.L., 2014. Effect of zinc oxide nanoparticles on cytology and seed germination in onion. International Journal of Current Microbiology and Applied Science 3, 467-473.

Raskin, I., Smith, R.D., Salt, D.E., 1997. Phytoremediation of metals: using plants to remove pollutants from the environment. Current Opinion in Biotechnology 8, 221-226.

Rathnayake, I.V.N., Megharaj, M., Krishnamurti, G.S.R., Bolan, N.S., Naidu, R., 2013. Heavy metal toxicity to bacteria – Are the existing growth media accurate enough to determine heavy metal toxicity? Chemosphere 90, 1195–1200.

Rauret, G., Lopez-Sanchez, J.F., Sahuquillo, A., Rubio, R., Davidson, C., Ure, A., Quevauviller, P.H., 1999. Improvement of the BCR three step sequential extraction procedure prior to the certification of new sediment and soil reference materials. Journal of Environmental Monitoring 1, 57 – 61.

Rauret, G., Lopez-Sanchez, J.F., Sahuquillo, A., Barahona, E., Lachica, M., Ure, A.M., Davidson, C.M., Gomez, A., Luck, D., Bacon, J., Yli-Halla, M., 2000. Application of a modified BCR sequential extraction (three-step) procedure for the determination of extractable trace metal contents in a sewage sludge amended soil reference material (CRM 483), complemented by a three-year stability study of acetic acid and EDTA extractable metal content. Journal of Environmental Monitoring 2, 228-233.

Rauser, W.E., 1995. Phytochelatins and related peptides: structure, biosynthesis, and function. Plant Physiology 109, 1141–1149.

Ravel, B., Newville, M., 2005. Athena, Artemis, Hephaestus: data analysis for X- ray absorption spectroscopy using IFEFFIT, Journal of Synchrotron Radiation 12, 537–541.

Rees, F., Germain, C., Sterckeman, T., Morel, J.L., 2015. Plant growth and metal uptake by a non-hyperaccumulating species (*Lolium perenne*) and a Cd-Zn hyperaccumulator (*Noccaea caerulescens*) in contaminated soils amended with biochar. Plant Soil 395, 57-73.

Reeve, W., O'Hara, G., Chain, P., Ardley, J., Bräu, L., Nandesena, K., Tiwari, R., Copeland, A., Nolan, M., Han, C., Brettin, T., 2010. Complete genome sequence of *Rhizobium leguminosarum bv. trifolii* strain WSM1325, an effective microsymbiont of annual Mediterranean clovers. Standards in Genomic Sciences 2, 347-356.

Reichman, S.M., 2002. The Responses of Plants to Metal Toxicity: A Review Forusing on Copper, Manganese and Zinc Melbourne: Australian Minerals and Energy Environment Foundation. pp. 22-26.

Reichman, S.M., 2007. The potential use of legume-rhizobium symbiosis for the remediation of arsenic contaminated sites. Soil Biology and Biochemistry 39, 2587-2593.

Reid, B.J, Jones, K.C., Semple, K.T., 2000. Bioavalability of persistent organic pollutants in soils and sediment- a perspective on mechanisms, consequences and assessment. Environmental Pollution 108, 103-112.

Reimer, L., 2013. Transmission electron microscopy: physics of image formation and microanalysis. Springer. pp.36.

Remedios, C., Rosario, F., Bastos, V., 2012. Environmental nanoparticles interactions with plants: morphological, physiological and genotoxic aspects. Journal of Botany 2012, 8.

Richards, J.W., Krumholz, G.D., Chval, M.S., Tisa, L.S., 2002. Heavy metal resistance patterns of Frankia strains. Applied Environmental Microbiology 68, 923–927.

Richards, L.A., Weaver, L.R., 1944. Moisture retention by some irrigated soils as related to soil moisture tension. Journal of Agricultural Research 69, 215-235.

Rickerby, D., Morrison, M., 2007. Report from workshop on nanotechnologies for environmental remediation, JRC Ispra. Available at: www.nanowerk.com/nanotechnology/reports/reportpdf/report101.pdf. [Accessed 2 April 2015].

Rico, C.M., Majumdar, S., Duarte-Gardea, M., Peralta-Videa, J.R., Gardea-Torresdey, J.L., 2011. Interaction of nanoparticles with edible plants and their possible implications in the food chain. Journal of Agricultural and Food Chemistry 59, 3485-3498.

Riding, M.J., Doick, K.J., Martin, F.L., Jones, K.C., Semple, K.T., 2013. Chemical measures of bioavailability/bioaccessibility of PAHs in soil: fundamentals to application. Journal of Hazardous Materials 261, 687-700.

Rieuwerts, J.S., Thornton, I., Farago, M.E., Ashmore, M.R., 1998. Factors influencing metal bioavailability in soils: preliminary investigations for the development of a critical loads approach for metals. Chemical Speciation and Bioavailability 10, 61-75.

Rieuwerts, J.S., 2007. The mobility and bioavailability of trace metals in tropical soils: a review. Chemical Speciation and Bioavailability 19, 75-85.

Rimmer, D.L., Vizard, C.G., Pless-Mulloli, T., Singleton, I., Air, V.S., Keatinge, Z.A., 2006. Metal contamination of urban soils in the vicinity of a municipal waste incinerator: one source among many. Science of the Total Environment 356, 207-216.

Ringli, C., 2005. The role of extracellular LRR-extensin (LRX) proteins in cell wall formation. Plant Biosystem 139, 32-35.

Rizwan, M., Ali, S., Qayyum, M.F., Ok, Y.S., Adrees, M., Ibrahim, M., Zia-ur-Rehman, M., Farid, M. and Abbas, F., 2017. Effect of metal and metal oxide nanoparticles on growth and physiology of globally important food crops: A critical review. Journal of hazardous materials 322, 2–16.

Roberts, A.P., Mount, A.S., Seda, B., Souther, J., Qiao, R., Lin, S., Ke, P. C., Rao, A.M., Klaine, S.J., 2007. In vivo biomodification of lipid-coated carbon nanotubes by *Daphnia magna*. Environment Science and Technology 41, 3025–3029.

Robinson, B., Russell, C., Hedley, M., Clothier, B., 2001. Cadmium adsorption by rhizobacteria: implication for New Zealand pastureland. Agricultural Ecosystem in the Environment 87, 315-321.

Robinson, B.H., Brooks, R.R., Howes, A.W., Kirkman, J.H., Greg, P.E.H., 1997. The potential of the high biomass nickel hyperaccumulator *Berkheya coddii* for phytoremediation and phytomining. Journal of Geochemical Exploration 60, 115-126.

Rodriguez, H., Gonzalez, T., Selman, G., 2000. Expression of a mineral phosphate solubilizing gene from *Erwinia herbicola* in two rhizobacterial strains. Journal of Biotechnology 84, 155–161.

Rodriguez, L., Ruiz, E., Alonso-Azcarate, J., Rincon, J., 2009. Heavy metal distribution and chemical speciation in tailings and soils around a Pb–Zn mine in Spain. Journal of Environmental Management 90, 1106-1116.

Rodriquez, M.G., Smith, I., 2003. Mechanism of iron regulation in mycobacteria: role in physiology and virulence. Molecular Microbiology 47, 1485-1495.

Rojas-Tapias, D., Moreno-Galvann, A., Pardo-Diaz, S., Obando, M., Rivera, D., Bonilla, R., 2012. Effect of inoculation with plant growth promoting bacteria (PGPB) on amelioration of saline stress in maize (*Zea mays*). Applied Soil Ecology 61, 264-272.

Rolfe, M.D., Rice, C.J., Lucchini, S., Pin, C., Thompson, A., Cameron, A.D.S., Alston, M., Stringer, M.F., Betts, R.B., Michael, J.B., Peck, W., Hinton, J.C.D., 2012. Lag phase is a distinct growth phase that prepares bacteria for exponential growth and involves transient metal accumulation. Journal of Bacteriology 194, 686–701.

Romeo, S., Francini, A., Ariani, A., Sebastiani, L., 2014. Phytoremediation of Zn: identify the diverging resistance, uptake and biomass production behaviours of poplar clones under high zinc stress. Water, Air and Soil Pollution 225, 1-12.

Romero, L.C., Aroca, M.A., Laureano-Marin, A.M., Moreno, I., Garcia, I., Goto, C., 2014. Cysteine and cysteine-related signalling pathways in *Arabidopsis thaliana*. Molecular Plant 7, 264–276.

Rosen, B., 2002. Biochemistry of arsenic detoxification. FEBS Letters 529, 86–92.

Roszak, D.B., Colwell, R.R., 1987. Survival strategies of bacteria in the natural environment. Microbiological Reviews 51, 365.

Roszak, J., Catalán, J., Järventaus, H., Lindberg, H.K., Suhonen, S., Vippola, M., Stępnik, M., Norppa, H., 2016. Effect of particle size and dispersion status on cytotoxicity and genotoxicity of zinc oxide in human bronchial epithelial cells. Mutation Research /Genetic Toxicology and Environmental Mutagenesis 805, 7-18.

Rout, G.R., Das, P., 2009. Effect of metal toxicity on plant growth and metabolism: zinc. Sustainable agriculture. Springer Netherlands. pp. 873-884.

Rout, G.R., Sahoo, S., 2015. Role of iron in plant growth and metabolism. Reviews in Agricultural Science 3, 1-24.

Ruby, M.V., Davis, A., Schoof, R., Eberle, S., Sellstone, C.M., 1996. Estimation of lead and arsenic bioavailability using a physiologically based extraction test. Environmental Science and Technology 30, 422-430.

Rupasinghe, R.A.T., 2011. Dissolution and aggregation of zinc oxide nanoparticles at circumneutral pH. A study of size effects in the presence and absence of citric acid. Master of Science, thesis, University of Iowa.

Sabo-Attwood, T., Unrine, J.M., Stone, J.W., Murphy, C.J., Ghoshroy, S., Blom, D., Bertsch, P.M., Newman, L.A., 2012. Uptake, distribution and toxicity of gold nanoparticles in tobacco (*Nicotiana xanthi*) seedlings. Nanotoxicology 6, 353-360.

Saeed, M., Fox, R.L., 1977. Relations between suspension pH and zinc solubility in acid and calcareous solls. Soil Science 124, 199-204.

Safronova, V.I., Stepanok, V.V., Engqvist, G.L, Alekseyev, Y.V., Belimov, A.A., 2006. Root-associated bacteria containing 1-aminocyclopropane-1-carboxylate deaminase

improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. Biology and Fertility of Soils 42, 267–272.

Sagardoy, R., Morales, F., López-Millán, A. F., Abadía, A., Abadía, J., 2009. Effects of zinc toxicity on sugar beet (*Beta vulgaris L.)* plants grown in hydroponics. Plant Biology 11, 339–350.

Saito, K., 2000. Regulation of sulfate transport and synthesis of sulfur-containing amino acids. Current Opinion in Plant Biology 3, 188–195.

Sajid, M., Ilyas, M., Basheer, C., Tariq, M., Daud, M., Baig, N., Shehzad, F., 2015. Impact of nanoparticles on human and environment: review of toxicity factors, exposures, control strategies, and future prospects. Environmental Science and Pollution Research 22, 4122-4143.

Sakakibara, M., Ohmori, Y., Ha, N.T.H., Sano, S., Sera, K., 2011. Phytoremediation of heavy metal contaminated water and sediment by *Eleocharis acicularis.* Clean Soil, Air, Water 39, 735–741.

Sakshi, S., Dhoble, S.J., Singh, P., Ramrakhiani, M., 2013. Fabrication of ZnS: Cu/PVA nanocomposite electroluminescence devices for flat panel displays. Advanced Materials Letters 4, 196-173.

Salt, D.E., Blaylock, M., Kumar, N.P., Dushenkov, V., Ensley, B.D., Chet, I., Raskin, I., 1995. Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Nature biotechnology 13, 468-474.

Salt, D.E., Pickering, I.J., Prince, R.C., Gleba, D., Dushenkov, S., Smith, R.D., Raskin, I., 1997. Metal accumulation by aquacultured seedlings of Indian mustard. Environmental Science and Technology 31, 1636-1644.

Salt, D.E., Smith, R.D., Raskin, I., 1998. Phytoremediation. Annual Review of Plant Physiology and Plant Molecular Biology 49, 643–668.

Salt, D.E., Prince, R.C., Baker, A.J.M., Raskin, I., Pickering, I. J., 1999. Zinc ligands in the metal hyperaccumulator *Thlaspi caerulescens* as determined using X-ray absorption spectroscopy. Environmental Science and Technology 33, 713–717.

Santiago-Martín, A.D., Constantin, B., Guesdon, G., Kagambega, N., Raymond, S., Cloutier, R.G., 2016. Bioavailability of engineered nanoparticles in soil systems. Journal of Hazardous, Toxic, and Radioactive Waste 20, 1.

Santoyo, G., Moreno-Hagelsieb, G., Orozco-Mosqueda, M.C., Glick, B.R., 2016. Plant growth-promoting bacterial endophytes. Microbiological Research 183, 92-99.

Sarawat, S., Rai, J.P.N., 2009. Phytoextraction potential of six plant species grown in multimetal contaminated soil. Chemistry and Ecology 25, 1-11.

Saraswat, S., Rai, J.P.N., 2011. Complexation and detoxification of Zn and Cd in metal accumulating plants. Reviews in Environmental Science and Biotechnology 10, 327-339.

Sarkar, A., Ghosh, M., Sil, P.C., 2014. Nanotoxicity: oxidative stress mediated toxicity of metal and metal oxide nanoparticles. Journal of Nanoscience and Nanotechnology 14, 730-743.

Sarret, G., Saumitou-Laprade, P., Bert, V., Proux, O., Hazemann, J.L., Traverse, A., Marcus, M.A., Manceau, A., 2002. Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiology 130, 1815-1826.

Sasaki, Y., Hayakawa, T., Inoue, C., Yukouka, T., 2006. Generation of mercury hyperaccumulating plants through transgenic expression of the bacterial mercury membrane transport protein MerC. Transgenic Research 15, 615–625.

Sastre, J., Sahuquillo, A., Vidal, M., Rauret, G., 2002. Determination of Cd, Cu, Pb and Zn in environmental samples: microwave-assisted total digestion versus aqua regia and nitric acid extraction. Analytica Chimica Acta 462, 59.

Sauve, S., Hendershot, W., Allen, H.E., 2000. Solid-solution partitioning of metals in contaminated soils: Dependence on pH, total metal burden, and organic matter. Environmental Science and Technology 34, 1125-1131.

Scheckel, K.G., Chaney, R.L., Basta, N.T., Ryan, J.A., 2009. Advances in assessing bioavailability of metal (loid) s in contaminated soils. Advances in Agronomy 104, 1-52.

Scheckel, K. G. Luxton, T. P. El Badawy, A. M. Impellitteri, C. A. Tolaymat, T. M., 2010. Synchrotron speciation of silver and zinc oxide nanoparticles aged in a kaolin suspension. Environmental Science and Technology 44, 1307–1312.

Scheinost, A.C., Kretzschmar, R., Pfister, S., Roberts, D.R., 2002. Combining selective sequential extractions, X-ray absorption spectroscopy, and principal component analysis for quantitative zinc speciation in soil. Environmental Science and Technology 36, 5021-5028.

Schwartz, C., Morel, J.L., Saumier, S., Whiting, S.N., Baker, A.J.M., 1999. Root development of the zinc-hyperaccumulator plant *Thlaspi caerulescens* as affected by metal origin, content and localization in soil. Plant Soil 208, 103–115.

Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A., Harms, H., 2004. Peer reviewed: defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. Environmental Science and Technology 38, 228A-231A.

SEPA., 2006. Slow progress on reclaiming Scotland's brownfield land: www. Scotland. gov. uk/Publications/2006/01/30155550/0.

Sepahvand, H., Forghani, A., 2012. Comparison of two sequential extraction procedures for the fractionation of zinc in agricultural calcareous soils. Chemical Speciation and Bioavailability 24, 13-22.

Seshadri, B., Bolan, N.S., Naidu, R., 2015. Rhizosphere-induced heavy metal (loid) transformation in relation to bioavailability and remediation. Journal of Soil Science and Plant Nutrition 15, 524-548.

Sessitsch, A., Kuffner, M., Kidd, P., Vangronsveld, J., Wenzel, W.W., Fallmann, K., Puschenreiter, M., 2013. The role of plant associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. Soil Biology and Biochemisrty 60, 182-194.

Seth, C.S., Chaturvedi, P.K., Misra, V., 2007. Toxic effect of arsenic and cadmium alone and in combination on Giant duckweed (*Spirodela polyrrhiza L*.) in response to its accumulation. Environmental Toxicology 22, 539–549.

Seth, C.S., Chaturvedi, P.K., Misra, V., 2008. The role of phytochelatins and antioxidants in tolerance to Cd accumulation in *Brassica juncea L.* Ecotoxicology Environmental Safety 71, 76–85.

Seth, C.S., 2012. A review on mechanisms of plant tolerance and role of transgenic plants in environmental clean-up. Botanical Review 78, 32–62.

Setia, R.C., Nayyar, H., Setia, N., 2008. Crop improvement strategies and application. I.K. International Publishing House, New Delhi, India. pp. 206-218.

Shaffer, R.J., Wallace, J., Garwood, F., 1937. The centrifuge method of investigating the variation of hydrostatic pressure with water content in porous material. Transactions of the Faraday Society 33, 723-734.

Shahid, M., Pinelli, E., Pourrut, B., Silvestre, J., Dumat, C., 2011. Lead-induced genotoxicity to *Vicia faba L.* roots in relation with metal cell uptake and initial speciation. Ecotoxicology and Environmental Safety 74, 78-84.

Shahid, M., Pinelli, E., Dumat, C., 2012. Review of Pb availability and toxicity to plants in relation with metal speciation; role of synthetic and natural organic ligands. Journal of Hazardous Materials 219, 1-12.

Shams, G., Ranjbar, M., Amiri, A.A., Khodarahmpour, Z., 2013. The effect of 35 nm silver nanoparticles on antagonistic and synergistic mineral elements in leaves and fruit of tomato (Lycopersicon esculentum Mill.). International Journal of Agriculture and Crop Sciences 5, 493-500.

Shan, X.Q., Wang, Z.W., Wang, W.S., Zhang, S.Z., Wen, B., 2003. Labile rhizosphere soil solution fraction for prediction of bioavailability of heavy metals and rare earth elements to plants. Analytical and Bioanalytical Chemistry 375, 400-407.

Shao, H.B., Chu, L.Y., Jaleel, C.A., Manivannan, P., Panneerselvam, R., Shao, M.A., 2009. Understanding water deficit stress-induced changes in the basic metabolism of higher plants—biotechnologically and sustainably improving agriculture and the

eco-environment in arid regions of the globe. Critical Reviews in Biotechnology 29, 131–151.

Sharma, A., Shankhdhar, D., Shankhdhar, S.C., 2014. Growth promotion of the rice genotypes by pgprs isolated from rice rhizosphere. Journal of Soil Science and Plant Nutrition 14, 505-517.

Sharma, D.M., Sharma, C.P., Tripathi, R.D., 2003. Phytotoxic lesions of chromium in maize. Chemosphere 51, 63-68.

Sharma, P., Pandey, S., 2014. Status of phytoremediation in world scenario. International Journal of Environmental Bioremediation and Biodegradation 2, 178-191.

Sharma, R., 2012. Studies on the thermoluminescence glow curves of Mn doped ZnS nanoparticles. Recent Research in Science and Technology 4, 34-35.

Sharma, S., Singh, B., Thulasidas, S.K., Kulkarni, M.J., Natarajan, V., Manchanda, V.K., 2016. Evaluation of terrestrial plants extracts for uranium sorption and characterization of potent phytoconstituents. International Journal of Phytoremediation 18, 10-15.

Sharma, V. K., Filip, J., Zboril, R., Varma, S.M., 2015. Natural inorganic nanoparticles – formation, fate, and toxicity in the environment. Chemical Society Review 44, 8410-8423.

Shaymurat, T., Gu, J., Xu, C., Yang, Z., Zhao, Q., Liu, Y., Liu, Y., 2012. Phytotoxic and genotoxic effects of ZnO nanoparticles on garlic (Allium sativum L.): A morphological study. Nanotoxicology 6, 241-248.

Sheng, X., Sun, L., Huang, Z., He, L., Zhang, W. and Chen, Z., 2012. Promotion of growth and Cu accumulation of bio-energy crop (Zea mays) by bacteria: implications for energy plant biomass production and phytoremediation. Journal of Environmental Management 103, 58-64.

Sheng, X.F., Xia, J.J., Jiang, C.Y., He, L.Y., Qian, M., 2008. Characterization of heavy metal-resistant endophytic bacteria from rape (*Brassica napus*) roots and their potential in promoting the growth and lead accumulation of rape. Environmental. Pollution 156, 1164-1170.

Shetty, K.G., Hetrick, B.A.D., Figge, D.A.H., Schwab, A.P., 1994. Effects of mycorrhizae and other soil microbes on revegetation of heavy metal contaminated mine spoil. Environmental Pollution 86, 181–188.

Shetty, K.G., Hetrick, B.A.D., Schwab, A.P., 1995. Effects of mycorrhizae and fertilizer amendments on zinc tolerance of plants. Environmental Pollution 88, 307-314.

Shuman, L.M., 1980. Zinc in soils. In zinc in environment, Part 1. Ecological Cycling. Ed. Nriagu, J.O. John Wiley and Sons, London. pp. 39-70.

Shuttleworth, K.L., Unz, R.F., 1991. Influence of metals and metal speciation on the growth of filamentous bacteria. Water resources. Water Research 25, 1177-1186.

Siddiqui, M.H., Al-Whaibi, M.H., Mohammad, F., 2015. Nanotechnology and plant sciences: Nanoparticles and their impact on plants. Springer.

Siddiqui, M.H., Al-Whaibi, M.H., Firoz, M., Al-Khaishany, M.Y., 2015. Role of nanoparticles in plants. In Nanotechnology and plant sciences. Ed. Siddiqui, M.H., Al-Whaibi, M.H., Firoz, M. Springer International Publishing. pp. 19-35.

Siedlecka, A., 2014. Some aspects of interactions between heavy metals and plant mineral nutrients. Acta Societatis Botanicorum Poloniae 64, 265-272.

Simonin, M., Richaume, A., 2015. Impact of engineered nanoparticles on the activity, abundance, and diversity of soil microbial communities: a review. Environmental Science and Pollution Research 22, 13710-13723.

Singer, A.C., Crowley, D.E., Thompson, I.P., 2003. Secondary plant metabolites in phytoremediation and biotransformation. Trends in Biotechnology 21, 123-130.

Singh, A. Fulekar, M.H., 2012. Phytoremediation of heavy metals by *Brassica juncea* in aquatic and terrestrial environment. The plant family *Brassicaceae*. Springer Netherlands. pp. 153-169.

Singh, A.K., Srivastava, S.C., Verma, P., Ansari, A., Verma, A., 2014. Hazard assessment of metals in invasive fish species of the Yamuna River, India in relation to bioaccumulation factor and exposure concentration for human health implications. Environmental Monitoring and Assessment 186, 3823-3836.

Singh, B.P., Upadhyay, R.K., Kumar, R., Yadav, K., Areizaga-Martinez, H.I., 2016. Infrared radiation assisted stokes' law based synthesis and optical characterization of zns nanoparticles. Advances in Optical Technologies, http://dx.doi.org/10.1155/2016/8230291.

Singh, J., Hembram, P., Basak, J., 2014. Potential of *Vigna unguiculata* as a phytoremediation plant in the remediation of Zn from Contaminated Soil. American Journal of Plant Sciences 5, 1156-1162.

Singh, S., Sinha, S., 2004. Morphoanatomical response of two varieties of *Brassica juncea (L.) Czern* grown on tannery sludge amended soil. Bulletin of Environmental Contamination and Toxicology 72, 1017-1024.

Singh, V.P., 2005. Metal toxicity and tolerance in plants and animals. Sarup and Sons. pp.328.

Sinha, S., Mukherjee, S.K., 2008. Cadmium- induced siderophore production by a high Cd-resistant bacteria strain relieved Cd toxicity in plants through root colonization. Current Microbiology 56, 55-60.

Smical, A.I., Hotea, V., Oros, V., Juhasz, J., Pop, E., 2008. Studies on transfer and bioaccumulation of heavy metals from soil into lettuce. Environmental Engineering and Management Journal 7, 609–615.

Smith, S., Ward, M., Lin, R., Brydson, R., Dall' Osto, M., Harrison, R., 2012. Comparative study of single particle characterization by transmission electron microscopy and time-of- flight aerosol mass spectrometry in the London atmosphere. Atomospheric Environment 62, 400-407.

Snapp, S.S., Aggarwal, V.D., Chirwa, R.M., 1998. Note on phosphorus andgenotype enhancement of biological nitrogen fixation and productivity of maize/bean intercrops in Malawi. Field Crops Research 58, 205-212.

Soenen, S.J., Parak, W.J., Rejman, J., Manshian, B., (Intra)Cellular stability of inorganic nanoparticles: effects on cytotoxicity, particle functionality, and biomedical applications. Chemical Review 115, 2109-2135.

Solanki, R., Dhankhar, R., 2011. Biochemical changes and adaptive strategies of plants under heavy metal stress. Biologia 66, 195-204.

Sole, V.A., Papillon, E., Cotte, M., Walter, P.H., Susini, J., 2007. A multiplatform code for the analysis of energy-dispersive X-ray fluorescence spectra. Spectrochimica Acta Part B 62, 63–68.

Sparks, D.L., 2003. Environmental soil chemistry. Academic press, Amsterdam, Netherlands.

Spencer, D., James, E.K., Ellis, G.J., Shaw, J.E., Sprent, J.I., 1994. Interactions between Rhizobia and potato tissue. Journal of Experimental Botany 45, 1475 – 1482.

Sposito, G., Lund, L., Chang, A., 1982. Trace metal chemistry in arid-zone field soils amended with sewage sludge: I. Fractionation of Ni, Cu, Zn, Cd, and Pb in solid phases. Soil Science Society of America Journal 46, 260–264.

Stark, W.J., Stoessel, P.R., Wohllebenb, W., Hafnerc, A., 2015. Industrial applications of nanoparticles. Chemical Society Review 44, 5793-5805.

Stephens, S.R., Alloway, B.J., Carter, J.E., Hodson, M.E., 2001. Changes in the leachabilituy of metals from degraded canal sediments during drying and oxidation. Environmental Pollution 114, 407-413.

Straczek, A., Sarret, G., Manceau, A., Hinsinger, P., Geoffroy, N., Jaillard, B., 2008. Zinc distribution and speciation in roots of various genotypes of tobacco exposed to Zn. Environmental and Experimental Botany 63, 80-90.

Ström, L., Owen, A.G., Godbold, D.L., Jones, D.L., 2002. Organic acid mediated P mobilization in the rhizosphere and uptake by maize roots. Soil Biology and Biochemistry 34, 703-710.

Su, D.C., Wong, J.W.C., 2004. Selection of mustard oilseed rape (*Brassica juncea L*.) for phytoremediation of cadmium contaminated soil. Bulletin of Environmental Contamination and Toxicology 72, 991-998.

Sun, R., Zhou, Q., Wei, S., 2011. Cadmium accumulation in relation to organic acids and non-protein thiols in leaves of the recently found cd hyperaccumulator *Rorippaglobosa* and the cd-accumulating plant *Rorippaislandica.* Journal of Plant Growth Regulation 30, 83–91.

Sun, Y., Zhou, Q., Wang, L., Liu, W., 2009. Cadmium tolerance and accumulation characteristics of *Bidens pilosa L*. as a potential Cd- hyperaccumulator. Journal of Hazardous Materials 161, 808-814.

Sun, Y., Zhou, Q., Xu, Y., Wang, L., Liang, X., 2011. The role of EDTA on cadmium phytoextraction in a cadmium hyperaccumulator *Rorippa globosa*. Journal of Environmental Chemistry and Ecotoxicology 3, 45-51.

Suriyaprabha, R., Karunakaran, G., Yuvakkumar, R., Prabu, P., Rajendran, V., Kannan, N., 2012. Growth and physiological responses of maize (Zea mays L.) to porous silica nanoparticles in soil. Journal of Nanoparticle Research 14, 1-14.

Sysalova, J., Szakova, J., 2006. Mobility assessment and validation of toxic elements in tunnel dust samples—Subway and road using sequential chemical extraction and ICP-OES/GF AAS measurements. Environmental Research 101, 287-293.

Taghavi, S., Garafola, C., Monchy, S., Newman, L., Hoffman, A., Weyens N., 2009. Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar. Applied and Environmental Microbiology 75, 748–757.

Takahashi, H., Kopriva, S., Giordano, M., Saito, K., Hell, R., 2011. Sulfur assimilation in photosynthetic organisms: molecular functions and regulations of transporters and assimilatory enzymes. Annual Review and Plant Biology 62, 157–184.

Talke, I.N., Hanikenne, M., Kramer, U., 2006. Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator Arabidopsis halleri. Plant Physiology 142, 148–167.

Tangahu, B.V., Abdullah, S.R.S., Basiri, H., Idris, M., Anuar, N., Mukhlisin, M., 2011. A review on heavy metals (As, Pb, and Hg) uptake by plants through phytoremediation. International Journal of Chemical Engineering 31.

Tank, N., Saraf, M., 2009. Enhancement of plant growth and decontamination of nickel-spiked soil using PGPR. Journal of Basic Microbiology 49, 195-204.

Tanwir, K., Akram, M.S., Masood, S., Chaudhary, H.J., Lindberg, S., Javed, M.T., 2015. Cadmium-induced rhizospheric pH dynamics modulated nutrient acquisition and physiological attributes of maize (*Zea mays L*.). Environmental Science and Pollution Research 22, 9193-9203.

Tao, S., Liu, W.X., Chen, Y.J., Xu, F.L., Dawson, R.W., Li, B.G., Cao, J., Wang, X.J., Hu, J.Y., Fang, J.Y., 2004. Evaluation of factors influencing root-induced changes of copper fractionation in rhizosphere of a calcareous soil. Environmental Pollution 129, 5–12.

Taranath, T.C., Patil, B.N., Santosh, T.U., Sharath, B.S., 2015. Cytotoxicity of zinc nanoparticles fabricated by Justicia adhatoda L. on root tips of Allium cepa L.—a model approach. Environmental Science and Pollution Research 22, 8611-8617.

Tavares, S., Wirtz, M., Beier, M.P., Bogs, J., Hell, R., Amâncio, S., 2015. Characterization of the serine acetyltransferase gene family of Vitis vinifera uncovers differences in regulation of OAS synthesis in woody plants. Frontiers in Plant Science 6, 74.

Templeton, A., Knowles, E., 2009. Microbial transformation of minerals and metals: recent advances in geomicrobiology derived from synchrotron-based x-ray spectroscopy and x-ray microscopy. Annual Review of Earth and Planetary Sciences 37, 367-391.

Terzano, R., Chami, Z.A., Vekemans, B., Janssens, K., Miano, T.M., Ruggiero, P., 2006. μ-XANES speciation of Zn in rhizospheric soil and in edible plants grown on a polluted soil amended with compost. HASYLAB Jahresbericht.

Terzano, R., Al Chami, Z., Vekemans, B., Janssens, K., Miano, T., Ruggiero, P., 2008. Zinc distribution and speciation within rocket plants (*Eruca vesicaria L. Cavalieri*) grown on a polluted soil amended with compost as determined by XRF microtomography and micro-XANES. Journal of Agricultural and Food Chemistry 56, 3222-3231.

Tessier, A., Campbell, P.G.C., Bisson, M., 1979. Sequential extraction procedure for the speciation of particulate trace metals. Analytical Chemistry 51, 844–851.

Thakur, S., Singh, L., Ab Wahid, Z., Siddiqui, M.F., Atnaw, S.M., Din, M.F.M., 2016. Plant-driven removal of heavy metals from soil: uptake, translocation, tolerance mechanism, challenges, and future perspectives. Environmental Monitoring and Assessment 188, 1-11.

Thottoli, A.K., Unni, A.K.A., 2013, Effect of trisodium citrate concentration on the particle growth of ZnS nanoparticles. Journal of Nanostructure in Chemistry 3, 56.

Tlustos, P., Szakova, J., Starkova, A., Pavlikovam, D., 2005. A comparison of sequential extraction procedures for fractionation of arsenic, cadmium, lead, and zinc in soil. Central European Journal of Chemistry 3, 830-851.

Tokala, R.K., Strap, J.L., Jung, C.M., Crawford, D.L., Salove, M.H., Deobald, L.A., Bailey, J.F. Morra, M.J., 2002. Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). Applied and Environmental Microbiology 68, 2161-2171.

Tosco, T., Papini, M.P., Viggi, C.C., Sethi, R., 2014. Nanoscale zero valent iron particles for groundwater remediation: a review. Journal of Cleaner Production 77, 1–12.

Tourinho, P.S., Van Gestel, C.A.M., Lofts, S., Svendsen, C., Soares, A.M.V., Loureiro, S., 2012. Metal-based nanoparticles in soil; Fate, Behaviour and effects on soil invertebrates. Environmental Toxicology and Chemistry 31, 1679-1692.

Trevors, J.T., Oddie, K.M., Belliveau, B.H., 1985. Metal resistance in bacteria. FEMS Microbiology Reviews 32, 39-54.

Turan, M., Esringu A., 2007. Phytoremediation based on canola (*Brassica napus L.*) and Indian mustard (*Brassica juncea L.)* planted on spiked soil by aliquot amount of Cd, Cu, Pb, and Zn. Plant Soil and Environment 53, 7–15.

Turkall, R. M., Abdel-Rahman, M., Skowronski, G.A., 2008. Effects of soil matrix and aging on the dermal bioavailability of hydrocarbons and metals in the soil: Dermal bioavailability of soil contaminants. Proceedings of the Annual International Conference on Soils, Sediments, Water and Energy 13, 29.

Turner, A.P., 1994. The responses of plants to heavy metals. In: Toxic metals in soil-plant systems. John Wiley and Sons, Chichester. pp. 153-187.

Twining, J.R., Payne, T.E., Itakura, T., 2004. Soil–water distribution coefficients and plant transfer factors for 134Cs, 85Sr and 65Zn under field conditions in tropical Australia. Journal of Environmental Radioactivity 71, 71-87.

Tyler, G., 2000. Effects of sample pretreatment and sequential fractionation by centrifuge drainage on concentrations of minerals in a calcareous soil solution. Geoderma 94, 59-70.

Uena, D., Zhao, F.G., Shen, R., Ma, J.F., 2004. Cadmium and zinc accumulation by the hyperaccumulator *Thlaspi caerulescens* from soils enriched with insoluble metal compounds. Soil Science and Plant Nutrition 50, 511-515.

UK Statutory Instrument.,1989. The Sludge (Use in Agriculture) Regulations 1989, no. 1263. Available at: http://www.legislation.gov.uk/uksi/1989/1263/made. [Accessed 02 January 2017].

Ummartyotin, S., Infahsaeng, Y., 2016. A comprehensive review on ZnS: From synthesis to an approach on solar cell. Renewable and Sustainable Energy Reviews 55, 17-24.

United Kingdom Environmental Agency., 2015. Environmental management – guidance: Contaminated land exposure assessment (CLEA) tool. Available at: https://www.gov.uk/government/publications/contaminated-land-exposure-assessment-clea-tool. [Accessed 22 November 2016].

Uren, N.C., Reisenauer, H.M., 1988. The role of root exudates in nutrient acquisition. Advances in plant nutrition. Praeger, New York. pp. 79–114.

USEPA., 1996. Ecological effects test guidelines (OPPTS 850.4200): Seed germination/root elongation toxicity test. EPA 712–C–96-154. Washington, DC.

USEPA., 2001. 4 0 CFR Part 745. Lead; identification of dangerous levels of lead; final rule. In Code of Fed Reg. pp. 1206-1240.

Vamerali, T., Bandiera, M., Mosca, G., 2010. Field crops for phytoremediation of metal contaminated land: a review. Environmental Chemistry Letters 8, 1–17.

Van Damme, A., Degryse, F., Smolders, E., Sarret, G., Dewit, R., Swennen, R., Manceau, A., 2010. Zinc speciation in mining and smelter contaminated overbank sediments by EXAFS spectroscopy. Geochimica et Cosmochimica Acta 74, 3707-3720.

Van Steveninck, R.F.M., Babare, A., Fernando, D.R., Van Steveninck, M.E., 1994. The binding of zinc, but not cadmium, by phytic acid in roots of crop plants. Plant Soil 167, 157–164.

Vangronsveld, J., Cunningham, S.D., 1998. Metal contaminated soils: In situ inactivation and phytorestoration. Springer-Verlag, Berlin. pp. 1-259.

Vara Prasad, M.N., De Oliveira Freitas, H.M., 2003. Metal hyperaccumulation in plants: biodiversity prospecting for phytoremediation technology. Electronic Journal of Biotechnology 6, 285-321.

Verma, J.P., Yadav, J., Tiwari, K.N., 2010. Application of Rhizobium sp. BHURC01 and plant growth promoting rhizobactria on nodulation, plant biomass and yields of chickpea (*Cicer arietinum L.*). International Journal of Agricultural Research 5, 148-156.

Verret, F., Gravot, A., Auroy, P., Leonhardt, N., David, P., Nussaume, L., Vavasseur, A., Richaud, P., 2004. Overexpression of *AtHMA4* enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Letters 576, 306–312.

Violante, A., Ricciardella, M., Pigna, M., Capasso, R., 2005**.** Effects of organic ligands on the sorption of trace elements onto metal oxides and organo-mineral complexes. Biogeochemistry of trace elements in the rhizosphere. Eds. Huang, P.M., Gordon, G.R. pp. 157-182.

Violante, A., Krishnamurti, G.S.R., Pigna, M., 2008. Mobility of trace elements in soil environments. In biophysico-chemical processes of metals and metalloids in soil environments. John Wiley and Sons, Hoboken, NJ. pp. 169-213.

Violante, A., Cozzolino, V., Perelomov, L., Caporale, A.G., Pigna, M., 2010. Mobility and bioavailability of heavy metals. Journal of Soil Science and Plant Nutrition 10, 268–292.

Voegelin, A., Pfister, S., Scheinost, A.C., Marcus, M.A., Kretzschmar, R., 2005. Changes in zinc speciation in field soil after contamination with zinc oxide. Environmental Science and Technology 39, 6616−6623.

Voegelin, A., Jacquat, O., Pfister, S., Barmettler, K., Scheinost, A.C., Kretzschmar, R., 2011. Time depedent changes of zinc speciation in four soils contaminated with zincite or sphalerite. Environmental Science and Technology 45, 255-261.

Vranova, V., Rejsek, K., Skene, K.R., Janous, D., Formanek, P., 2013. Methods of collection of plant root exudates in relation to plant metabolism and purpose: A review. Journal of Plant Nutrition and Soil Science 176, 175-199.

Waalewinjin-Kool, P.L.W., Ortiz, M.D., Lofts, S., Van Gestel, C.A.M., 2013a. The effect of pH on the toxicity of zinc oxide nanoparticles to *Folsomia Candida* in amended field soil. Environmental Toxicology and Chemistry 32, 2349-2355.

Waalewijin-Kool, P.L., Ortiz, M, Van Straalen, N.M., Van Gestel, C.A.M., 2013b. Sorption, dissolution and pH determine the long-term equilibration and toxicity of coated and uncoated ZnO nanoparticles in soil. Environmental Pollution 178, 59–64.

Wali, A., Colinet, G., Ksibi, M., 2014. Speciation of heavy metals by modified BCR sequential extraction in soils contaminated by phosphogypsum in Sfax, Tunisia. Environmental Research, Engineering and Management 70, 14-26.

Wang, B., Liu, L., Gao, Y., Chen, J., 2009. Improved phytoremediation of oilseed rape (*Brassica napus*) by *Trichoderma* mutant constructed by restriction enzyme - mediated integration (REMI) in cadmium polluted soil. Chemosphere 74, 1400–1403.

Wang, D., Lin, Z., Wang, T., Yao, Z., Qin, M., Zheng, S., Lu, W., 2016. Where does the toxicity of metal oxide nanoparticles come from: The nanoparticles, the ions, or a combination of both? Journal of Hazardous Materials 308, 328-334.

Wang, J., Feng, X., Anderson, C.W.N., Xing, Y., Shang, L., 2012. Remediation of mercury contaminated sites –A review. Journal of Hazardous Materials 221, 1-18.

Wang, P., Menzie, N.W., Lombi, E., McKenna, B.A., Johannessen, B., Glover, C.J., Kappen, P., Kopittke, P.M., 2013. Fate of ZnO nanoparticles in soils and cowpea (*Vigna unguiculata*). Environmental Science and Technology 47, 13822-13830.

Wang, S., Jia, Y., Wang, X., Wang, H., Zhoa, Z., Liu, B., 2010. Fractionation of heavy metals in shallow marine sediments from Jinzhou, China. Journal of Environmental Sciences 22, 23-31.

Wani, P.A., Khan, M.S., Zaidi, A., 2008. Effect of metal-tolerant plant growth-promoting rhizobium on the performance of pea grown in metal-amended soil. Archives of Environmental Contamination and Toxicology 55, 33–42.

Wani, P.A., Khan, M.S., 2010. Bacillus species enhance growth parameters of chickpea (*Cicer arietinum L.*) in chromium stressed soils. Food and Chemical Toxicology 48, 3262-3267.

Watt, M., Silk, W.K., Passioura, J.B., 2006. Rates of root and organism growth, soil conditions, and temporal and spatial development of the rhizosphere. Annals of Botany 97, 839–855.

Webb, S.M., Leppard, G.G., Gaillard, J.F., 2000. Zinc speciation in a contaminated aquatic environment: characterization of environmental particles by analytical electron microscopy. Environmental Science and Technology 34, 1926-1933.

Wei, L., Luo, C., Li, X., Shen, Z., 2008. Copper accumulation and tolerance in *chrysanthemum coronarium L* and *Sorghum Sudanese L*. Archives of Environmental Contamination and Toxicology 55, 238-246.

Wei, S., Teixeira da Silva, J.A., Zhou, Q., 2008. Agro-improving method of phytoextracting heavy metal contaminated soil. Journal of Hazardous Materials 150, 662-668.

Wenzel, W.W., Bunkowski, M., Puschenreiter, M., Horak, O., 2003. Rhizosphere characteristics of indigenously growing nickel hyperaccumulator and tolerant plants on serpentine soil. Environmental Pollution 123, 131-138.

Wenzel, W.W., 2009. Rhizosphere processes and management in plant-assisted bioremediation (phytoremediation) of soils. Plant Soil 321, 385-408.

Weyens, N., Van der Lelie, D., Taghavi, S., Newman, L., Vangronsveld, J., 2009 a. Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends in Biotechnology 27, 591–598.

Weyens, N., Van der Lelie, D., Taghavi, S., Vangronsveld, J., 2009b. Phytoremediation: plant–endophyte partnerships take the challenge. Current Opinion in Biotechnology 20, 248–254.

White, R.E., 2013. Principles and practice of soil science: the soil as a natural resource. John Wiley and Sons.

Whiting, S.N., de Souza, M.P., Terry, N., 2001b. Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environmental Science and Technology 35, 3144-3150.

Whiting, S.N., Leake, J.R., McGrath, S.P., Baker, A.J., 2001a. Zinc accumulation by *Thlaspi caerulescens* from soils with different Zn availability: a pot study. Plant and Soil 236, 11-18.

Wiehe, W., Holfich, G., 1995. Survival of plant growth promoting rhizosphere bacteria in rhizosphere of different crops and migration to non-inoculated plants under field conditions innorth-east Germany. Microbiology Research 150, 201-206.

Wierzbicka, M., Obidzinska, J., 1998. The effect of lead on seed imbibition and germination in different plant species. Plant Science 137, 155-171.

Wilkinson, K.J., Reinhardt, A., 2005. Contrasting roles of natural organic matter on colloidal stabilization and flocculation in freshwaters. Flocculation in Natural and Engineered Environmental Systems. CRC Press, Boca Raton, FL. pp. 143–170.

Wilson, S.M., Bacic, A., 2012. Preparation of plant cells for transmission electron microscopy to optimize immunogold labeling of carbohydrate and protein epitopes Nature Protocols 7, 1716–1727.

Wintz, H., Fox, T., Wu, Y.Y., Feng, V., Chen, W., Chang, H.S., Zhu, T., Vulpe, C., 2003. Expression profiles of *Arabidopsis thaliana* in mineral deficiencies reveal novel transporters involved in metal homeostasis. Journal of Biological Chemistry 278, 47644-47653.

Wojcik, M., Vangronsveld, J., Tukiendorf, A., 2005. Cadmium tolerance in *Thlaspi caerulescens*-II. Localization of cadmium in *Thlaspi caerulescens*. Environmental Experimental Botany 53, 163-171.

Wong, C.K.E., Cobbett, C.S., 2009. HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in Arabidopsis thaliana. New Phytologist 181, 71–78.

Wong, M.H., Bradshaw, A.D., 1982. A comparison of the toxicity of heavy metals using elongation of ryegrass, *Lolium perenne*. New Phytologist 91, 255–261.

Wu, B., Wang, Y., Lee, Y.H., Horst, A., Wang, Z.P., Chen, D.R., Sureshkumar, R., Tang, Y.J.J., 2010. Comparative eco-toxicities of nano-ZnO particles under aquatic and aerosol exposure modes. Environmental Science and Technology 44, 1484- 1489.

Wu, L. H., Luo, Y.M., Xing, X.R., Christie P., 2004. EDTA-enhanced phytoremediation of heavy metal contaminated soil with Indian mustard and associated potential leaching risk. Agriculture Ecosystems and Environment 102, 307–318.

Wu, S.C., Cheung, K.C., Luo, Y.M., Wong, M.H., 2006. Effects of inoculation of plant growth–promoting rhizobacteria on metal uptake by *Brassica juncea.* Environmental Pollution 140, 124-135.

Xiong, L.M., Lu, R.K., 1993. Effect of liming on plant accumulation of cadmium under upland or flooded conditions. Environmental Pollution 79, 199–203.

Xu, J., Kleja, D.B., Biester, H., Lagerkvist, A., Kumpiene, J., 2014. Influence of particle size distribution, organic carbon, pH and chlorides on washing of mercury contaminated soil. Chemosphere 109, 99-105.

Xu, M., Fujita, D., Kajiwara, S., Minowa, T., Li, X., Takemura, T., Iwai, H., Hanagata, N., 2010. Contribution of physicochemical characteristics of nano-oxides to cytotoxicity. Biomaterials 31, 8022–8031.

Xu, X., Hu, L., Gao, N., Liu, S., Wageh, S., Al-Ghamdi, A.A., Alshahrie, A., Fang, X., 2015. Controlled growth from ZnS nanoparticles to ZnS–CdS nanoparticle hybrids with enhanced photoactivity. Advanced Functional Materials 25, 445-454.

Yadaf, S.K., Juwarkar, A.A., Kumar, G.P., Thawale, P.R., Singh, S.K., Chakrabari, T., 2009. Bioaccumulation and phyto-translocation of arsenic, chromium and zinc by *Jatropha cursa L*.: Impact of diary sludge and biofertilizer. Bioresource Technology 100, 4616-4622.

Yadav, S.K., 2010. Heavy metal toxicity in plants: an overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. South African Journal of Botany 76, 167–179.

Yamabi, S., Imai, H., 2002. Growth conditions for wurtzite zinc oxide films in aqueous solutions. Journal of Material Chemistry 12, 3773-3778.

Yang, H., Liu, C., Yang, D., Zhang, H., Xi. Z., 2009. Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition. Journal of Applied Toxicology 29, 69–78.

Yang, L., Watts, D.J., 2005. Particle surface characteritics may play an important role in phytotoxicity of alumina nanoparticles. Toxicology Letters 158, 122-132.

Yang, X., Feng, Y., He, Z., Stoffella P. J., 2005. Molecular mechanisms of heavy metal hyperaccumulation and phytoremediation. Journal of Trace Elements in Medicine and Biology 18, 339–353.

Yang, X.W., Tian, X.H., Lu, X.C., Cao, Y.X., Chen, Z.H., 2011. Impacts of phosphorus and zinc levels on phosphorus and zinc nutrition and phytic acid concentration in wheat (*Triticum aestivum L*.). Journal of the Science of Food and Agriculture 91, 2322-2328.

Yang, Y., Wang, Y., Westerhoff, P., Hristovski, K., Jin, V.L., Johnson, M.V.V., Arnold, J.G., 2014. Metal and nanoparticle occurrence in biosolid-amended soils. Science of the Total Environment 485, 441-449.

Yang, Z., Chen, J., Dou, R., Gao, X., Mao, C., Wang, L., 2015. Assessment of the phytotoxicity of metal oxide nanoparticles on two crop plants, maize (*Zea mays l*.) and rice (*Oryza sativa L*.). International Journal of Environmental Research and Public Health 12, 15100-15109.

Yi, H., Dey, S., Kumaran, S., Lee, S.G., Krishnan, H.B., Jez, J.M., 2013. Structure of soybean serine acetyltransferase and formation of the cysteine regulatory complex as an enzyme chaperone. Journal of Biology Chemistry 288, 36463-36472.

Yousaf, S., Ripka, K., Reichenauer, T.G., Andria, V., Afzal, M., Sessitsch, A., 2010. Hydrocarbon degradation and plant colonization by selected bacterial strains isolated from Italian ryegrass and birdsfoot trefoil. Journal of Applied Microbiology 109, 1389–1401.

Yu, J., Lv, X., Bin, M., Wu H., Du, S., Zhou, M., Yang, Y., Han, G., 2015. Fractal features of soil particle size distribution in newly formed wetlands in the Yellow River Delta. Scientific Reports 5, 10540.

Yuan, C.G., Shi, J.B., He, B., Liu, F.J., Liang, L.N., 2004. Speciation of heavy metals in marine sediments from the East China Sea by ICP- MS with sequential extraction. Environment International 30, 769-783.

Zafar, H., Ali, A., Ali, J.S., Haq, I.U., Zia, M., 2016. Effect of ZnO nanoparticles on *Brassica nigra* seedlings and stem explants: growth dynamics and antioxidative response. Frontiers in Plant Science 7, 535.

Zaidi, S., Usmani, S., Singh, B.R., Musarrat, J., 2006. Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere 64, 991-997.

Zaurov, D.E., Perdomo, P., Raskin, I., 1999. Optimizing soil fertility and pH to maximize cadmium removed by Indian mustard from contaminated soils. Journal of Plant Nutrition 22, 977–98.

Zayed, A., Gowthaman, S., Terry, N., 1998. Phytoaccumulation of trace elements by wetland plants: I. Duckweed. Journal of Environmental Quality 27, 715–721.

Zeng, F., Ali, S., Zhang, H., Ouyang, Y., Qiu, B., Wu, F., Zhang, G., 2011. The influence of pH and organic matter content in paddy soil on heavy metal availability and their uptake by rice plants. Environmental Pollution 159, 84-91.

Zeng, W.X., Ma, L.Q., Qiua, R.L., Tanga, Y.T., 2011. Effects of Zn on plant tolerance and non-protein thiol accumulation in Zn hyperaccumulator *Arabis paniculata Franch.* Environmental and Experimental Botany 70, 227–232

Zeng, X., Ma, L.Q., Qui, R., Tang, Y., 2009. Responses of non –protein thiols to Cd exposure in Cd hyperaccumulator *Arabis Paniculata Franch*. Environmental and Experimental Botany 66, 242-248.

Zeng, X.W., Ma, L.Q., Qiu, R.L., Tang, Y.T., 2011. Effects of Zn on plant tolerance and non-protein thiol accumulation in Zn hyperaccumulator *Arabis paniculata Franch*. Environmental and Experimental Botany 70, 227–232.

Zhang, C., Song, N., Zeng, G.M., Jiang, M., Zhang, J.C., Hu, X.J., Chen, A.W., Zhen, J.M., 2014. Bioaccumulation of zinc, lead, copper, and cadmium from contaminated sediments by native plant species and Acrida cinerea in South China. Environmental Monitoring and Assessment 186, 1735-1745.

Zhang, D., Hua, T., Xiao, F., Chen, C., Gersberg, R., Liu, Y., Stuckey, D., Ng, W, J., Tan, S.K., 2015. Phytotoxicity and bioaccumulation of ZnO nanoparticles in *Schoenoplectus tabernaemontani*. Chemosphere 120, 211-219.

Zhang, J., Yang, J.C., Wang, R.Q., Hou, H., Du, X.M., Fan, S.K., Liu, J.S., Dai, J.L., 2013. Effects of pollution sources and soil properties on distribution of polycyclic aromatic hydrocarbons and risk assessment. Science of the Total Environment 463, 1-10.

Zhang, L., Jiang, Y., Ding, Y., Povey, M., York, D., 2007. Investigation into the antibacterial behaviour of suspensions of ZnO nanoparticles (ZnO nanofluids). Journal of Nanoparticle Research 9, 479-489.

Zhang, P., Ma, Y., Zhang, Z., He, X., Zhang, J., Guo, Z., Tai, R., Zhao, Y., Chai, Z., 2012. Biotransformation of ceria nanoparticles in cucumber plants. ACS Nano 6, 9943-9950.

Zhang, P., Ma, Y., Zhang, Z., 2015. Interactions between engineered nanomaterials and plants: phytotoxicity, uptake, translocation, and biotransformation. Nanotechnology and Plant Sciences. Springer International Publishing. pp 77-99.

Zhang, R., Zhang, H., Tu, C., Hu, X., Li, L., Luo, Y., Christie, P., 2015. Phytotoxicity of ZnO nanoparticles and the released Zn (II) ion to corn (*Zea mays L.)* and cucumber (*Cucumis sativus* L.) during germination. Environmental Science and Pollution Research 22, 11109-11117.

Zhang, X., Xia, H., Li, Z., Zhang, P., Gao, B., 2010. Potential of four forage grasses in remediation of Cd and Zn contaminated soils. Bioresources Technology 101, 2063-2066.

Zhang, Y.F., He, L.Y., Chen, Z.J., Wang, Q.Y., Qian, M., Sheng, X.F., 2011a. Characterisation of ACC deaminase producing endophytic bacteria isolated from copper-tolerant plants and their potential in promoting the growth and copper accumulation of *Brassica napus.* Chemosphere 83, 57-62.

Zhang, Y.F., He, L.Y., Chen, Z.J., Zhang, W.H., Wang, O.Y., Qian, M., Sheng, X.F., 2011b. Characteristics of lead-resistant and ACC deaminase-producing endophytic bacteria and their potential in promoting lead accumulation of rape. Journal of Hazardous Materials 186, 720-725.

Zhao, L., Yuan, L., Wang, Z., Lei, T., Yin, X., 2012. Phytoremediation of zinc-contaminated soil and zinc-biofortification for human nutrition. Phytoremediation and Biofortification. Springer Netherlands. pp. 33-57.

Zhao, L., Hernandez-Viezcas, J.A., Perlata-Videa, J.R., Peng, B., Munoz, B., Keller, A. A., Gardea-Torresdey, J.L., 2013a. ZnO nanoparticle fate in soil and zinc bioaccumulation in cornplants (Zea mays) influenced by alginate. Environmental Science: Processes and Impact 15, 260−266.

Zhao, L., Peralta-Videa, J.R., Rico, C.M., Hernandez-Viezcas, J.A., Sun, Y., Niu, G., Servin, A., Nunez, J.E., Duarte-Gardea, M., Gardea-Torresdey, J.L., 2014. CeO_2 and ZnO nanoparticles change the nutritional qualities of cucumber (Cucumis sativus). Journal of Agricultural and Food Chemistry 62, 2752-2759.

Zheljazkov, V.D., Warman, P.R., 2004. Phytoavailability and fractionation of copper, manganese, and zinc in soil following application of two composts to four crops. Environmental Pollution 131, 187-195.

Zhou, T., Chen, D., Sun, Q., Li, L., Liu, F., Shen, Q., Shen, B., 2012. Isolation and characterization of *Pseudomonas brassicacearum* J12 as an antagonist against *Ralstonia solanacearum* and identification of its antimicrobial components. Microbiological Research 167, 388-394.

Zhu, X., Tian, S., Wang, C., Zhao, L., Zhou, J., Cai, Z., 2016. Ecotoxicological risk of engineered nanomaterials (ENMS) for the health of the marine environment. Engineered Nanoparticles and the Environment: Biophysicochemical Processes and Toxicity. pp. 442.

Zhuang, X., Chen, J., Shim, H., Bai, Z., 2007. New advances in plant growth-promoting rhizobacteria for bioremediation. Environment International 33, 406-413.